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COMMUNICABLE DISEASE AND IMMUNOLOGY,
INTERNAL MEDICINE, NUCLEAR MEDICINE,
PHYSIOLOGY, PSYCHIATRY, SURGERY AND
VETERINARY MEDICINE. VOLUME II

Walter Reed Army Institute of Research
Washington, D. C.

30 June 1973

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Annual Progress Report
1 July 1972 - 30 June 1973

Volume II

Walter Reed Army Institute of Research
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FOREWORD

In conducting the research described in this report, the investigators adhered to the "Guide for Laboratory Animal Facilities and Care," as promulgated by the Committee on the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, National Academy of Sciences - National Research Council.

SUMMARY

The various subjects covered in this report are listed in the Table of Contents. Abstracts of the individual investigations are included on the DD Form 1498 introducing each work unit report, and names of investigators are given at the beginning of each report.

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24. (U) Routine diagnostic, epidemiological, serological, and psychological procedures are being utilized.						
25. (U) 72 08 - 73 06 Virus research included epidemiological studies on dengue fever, Japanese 13 encephalitis, viral influenza and hepatitis B antigen. Gonorrhea was reported in asymptomatic Thai females; penicillinase producing staphylococci was found in 81 percent of male gonorrhea and an outbreak of meningococcal meningitis was reported. Parasitic diseases studied included malaria and gnathostomiasis. Studies continued on veterinary diseases, and mortality in laboratory rodents and sub-human primates was analyzed. A series of questions which may potentially discriminate US soldiers who are high risk for drug abuse has been identified. A method for screening urine for methaqualone was developed. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 72-30 Jun 73.						

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Work Unit 002 Tropical and subtropical military medical research

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A. DENGUE HEMORRHAGIC FEVER

1. Catabolic Rates of C3 and Clq of Patients with Dengue Hemorrhagic Fever

A small but important proportion of children experiencing secondary dengue virus infections develop dengue hemorrhagic fever (DHF) and its most severe form, dengue shock syndrome (DSS). Principal manifestations of DSS are hypovolemic shock, increased vascular permeability, mild to moderate disseminated intravascular coagulation, and hemorrhage. Previous observations of marked depressions of C3 levels during acute DSS suggested complement activation as an immunopathogenic mechanism. In 1971 a study to

define the role of the complement system in the immunopathogenesis of DHF was carried out in Bangkok (SMRL Annual Report, 1972 p. 75-87). Studied were 55 patients with DHF, 36 of whom had DSS. Serum concentrations of 9 complement proteins, transferrin, fibrinogen and fibrin-split products were measured by radial immunodiffusion. The concentration of all measured complement components with the exception of C9 were depressed during shock. Concomitant but lesser reductions of the non-complement protein transferrin, however, suggested that depressions of complement proteins were at least in part due to extravasation from the vascular compartment. There was an inverse correlation between serum complement levels and grade of disease. C3 and C5 concentrations were most affected and were reduced to 20-40% of normal in severe DSS cases. Activation of both known complement pathways was indicated by simultaneous depression of C4 and C3 proactivator levels. Decrease of plasma fibrinogen, appearance of fibrin-split products, and severe thrombocytopenia during shock indicated occurrence of disseminated intravascular coagulation.

Low levels of serum complement components in DSS could be due to 1) increased consumption, 2) decreased synthesis and/or 3) sequestration of protein extravascularly due to increased vascular permeability. In order to confirm the strong but circumstantial evidence of complement component consumption in DSS found in the 1971 study, catabolic studies of 2 complement proteins, C3 and Clq, were undertaken in DHF patients in 1972.

Metabolic studies using ^{125}I -C3 or ^{125}I -labeled Clq were performed on 23 DHF patients. Patients with non-acute, non-infectious diseases served as controls. The degree of extravasation of serum proteins during shock was assessed by injecting ^{131}I -IgG as a marker simultaneously with ^{125}I -C3 or ^{125}I -Clq. In both studies, thyroid uptake of radioactive I was blocked by preliminary and continuing administration of saturated potassium iodide.

In the C3 metabolic studies $15\text{ }\mu\text{C}$ of ^{125}I -C3 and $15\text{ }\mu\text{C}$ of ^{131}I -IgG were inoculated intravenously. Eight blood specimens were obtained during the day of inoculation and two blood specimens were obtained on days 2-8 post inoculation. Determinations of total radioactivity for ^{125}I and ^{131}I , immunochemical quantitation of all complement proteins, transferrin, fibrinogen, and hemopexin were performed on all samples.

In Clq metabolic studies, $15\text{ }\mu\text{C}$ of ^{125}I -Clq and $15\text{ }\mu\text{C}$ of ^{131}I -IgG were inoculated intravenously. Blood was drawn at 1 minute, 10 minutes, 30 minutes, 1 hour, 3 hours, 6 hours, 24 hours,

36 hours, 48 hours and 60 hours after injection for determination of ^{125}I and ^{131}I radioactivity and immunochemical quantitation of serum components as in the C3 studies.

Serum samples obtained daily during the study from patients were tested for HI antibody to dengue 1-4, JEV, and Chikungunya antigens. Patients with 4-fold or greater titer rises to at least one dengue antigen or fixed titers $\geq 1:640$ to two or more dengue antigens were considered to have dengue infections.

Sequential levels of C3, C5, and transferrin and the platelet counts of one DSS patient studied are shown in Figure 1. In this patient (as with most studied), transferrin was lowered concomitantly with levels of C3 and C5, although to a much lesser degree. This example demonstrates the difficulty in assessing qualitatively or quantitatively complement activation by determining sequential serum levels of complement proteins.

In the catabolic studies, extravasation was assessed by injecting radio-labeled IgG simultaneously with C3. The left half of Figure 2 shows the elimination of IgG and C3 in a normal individual; the right half of Figure 2 shows the elimination of these proteins in patient with severe (grade IV) DSS. Within the first 20 hours of study, approximately 85% of the injected IgG was eliminated. That a sizeable proportion of IgG eliminated was pooled extravascularly is indicated by the increase of percentage of injected IgG recovered from 20-110 hours after injection (mobilization of extravasated IgG back into the vascular compartment after the shock period). By comparing the measured values of IgG of each patient with the expected normal values, a factor was obtained which served to correct C3 values for the amount of extravasation.

Figure 3 compares the elimination of C3 in the same DSS and control patient after correction for extravasation. The difference in the rate of elimination occurred within the first 20-30 hours. Thereafter both individuals eliminated C3 at the same rate. The initial rapid phase of C3 elimination coincided with shock in this patient.

Figure 4 compares the elimination of radio-labeled Clq in a patient with grade II DHF with that of a normal control. For the Clq values depicted, similar corrections as for C3 were made; radio-labeled IgG was injected simultaneously with Clq and IgG values were used to correct for extravasation. A vast difference in Clq elimination between these individuals is evident with a more rapid catabolic rate in the DHF patient.

Turnover studies of C3 were performed on 17 patients and 6 controls. Studies of Clq catabolism were performed on 7 patients and 3 controls. Figure 5 summarizes results of the C3 study. The fractional catabolic rate of C3 correlated well with serum C3 levels and the grade of illness. Eleven patients with DSS (grade III-IV) eliminated 2.6-3.5% of the C3 plasma pool per hour during a seven day observation period, whereas 5 patients with DHF without shock eliminated only 1.9-2.6%. The average of 5 controls was 1.9%. The greatest increase in catabolic rate of C3 was observed during the initial 24-48 hour period of study which coincided with shock.

The catabolic rate of Clq in the 7 dengue patients studied was increased over that of control individuals (Figure 6). During the 3 day observation period, the DHF patients eliminated 3.8-8.3% of their Clq plasma pool per hour, while control individuals eliminated 2.8-3.5% per hour. There was no apparent correlation between catabolic rate and grade of disease in the 7 patients; this was expected since both complement pathways (classical, involving Clq and the bypass mechanism, not involving Clq) are activated in DHF.

The results lend support to the concept that activation of complement constitutes an essential part of the pathogenic mechanism of DSS and DHF. Complement-dependent release of vasoactive amines and generation of platelet procoagulant activity are envisaged as major pathogenic factors of the dengue shock syndrome.

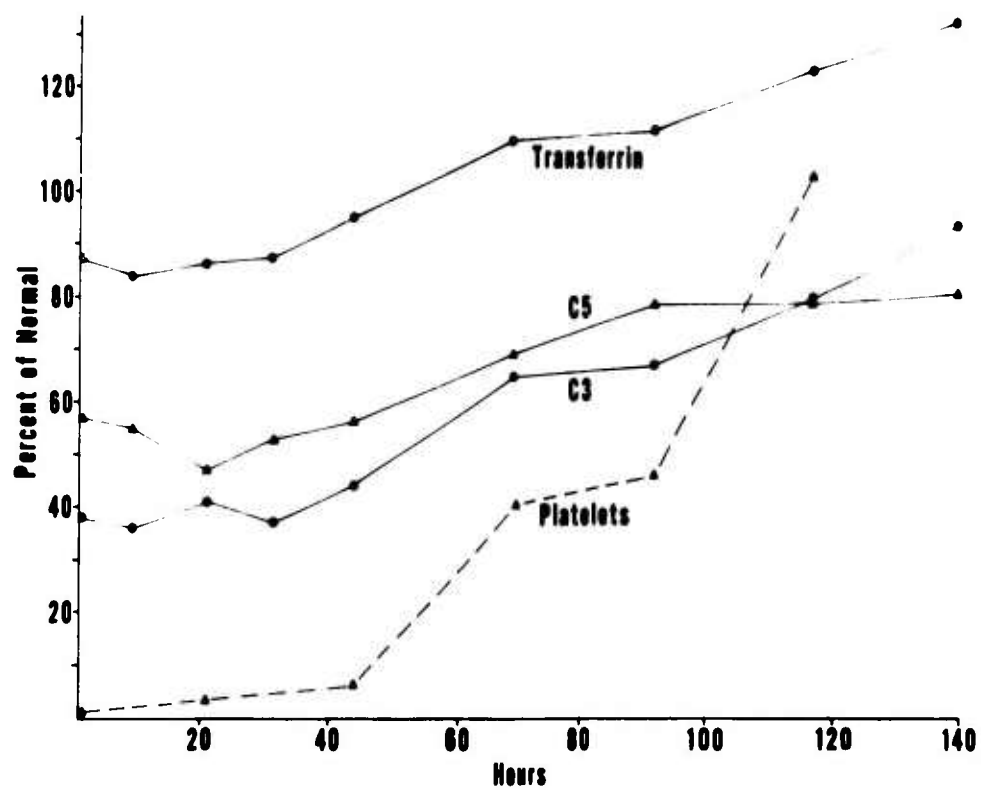


Figure 1. Sequential Plasma Concentrations of C3, C5, and Transferrin and Platelet Counts in a Patient with DSS

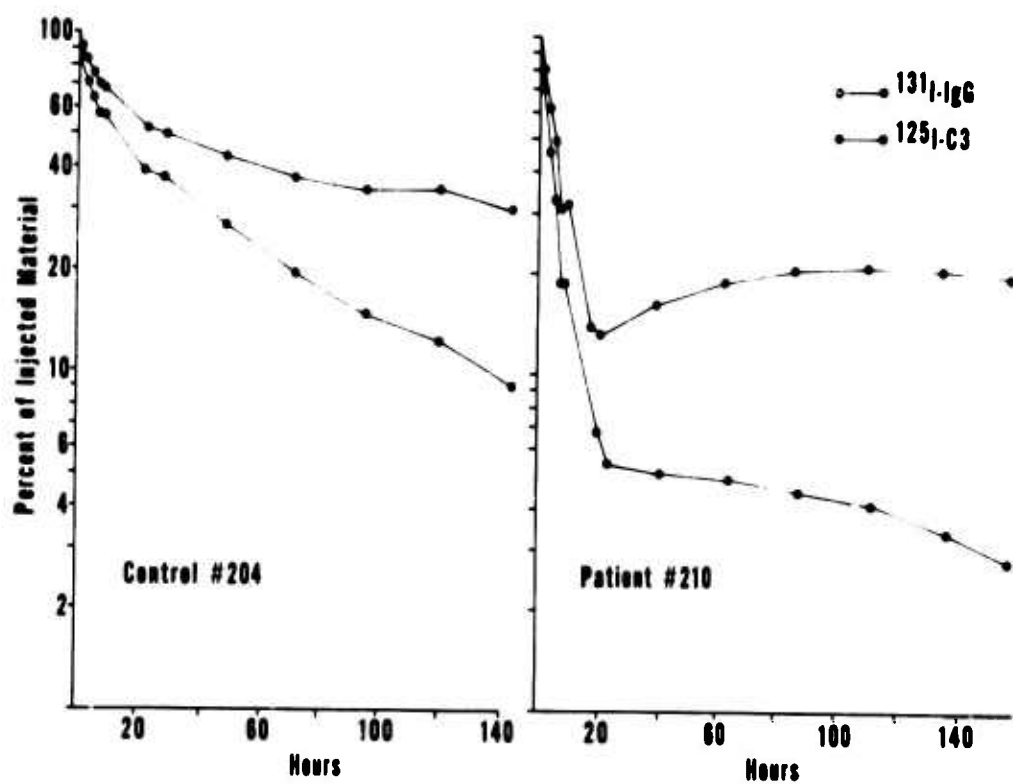


Figure 2. Disappearance of Injected ^{125}I C3 and ^{131}I IgG in a Normal Control and DSS Patient

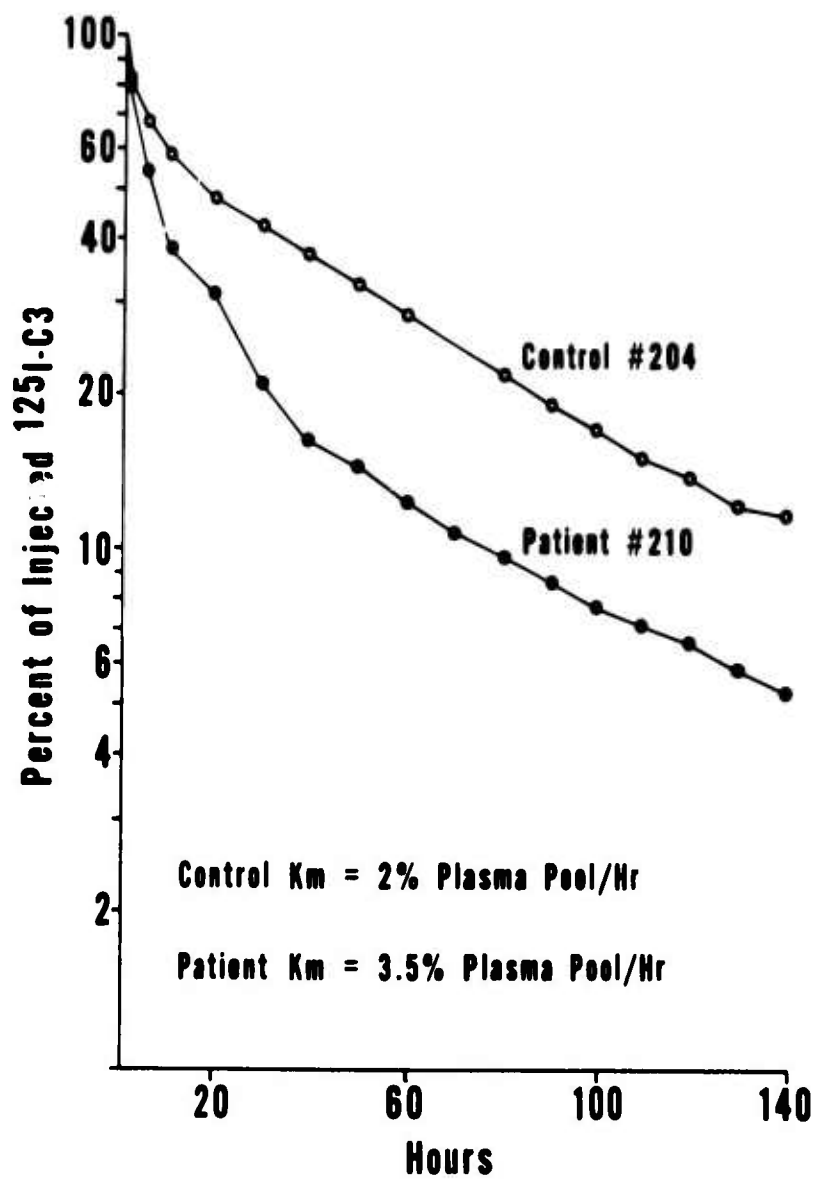


Figure 3. Corrected Elimination Rates of $^{125}\text{I C3}$ in a Normal Control and DSS Patient

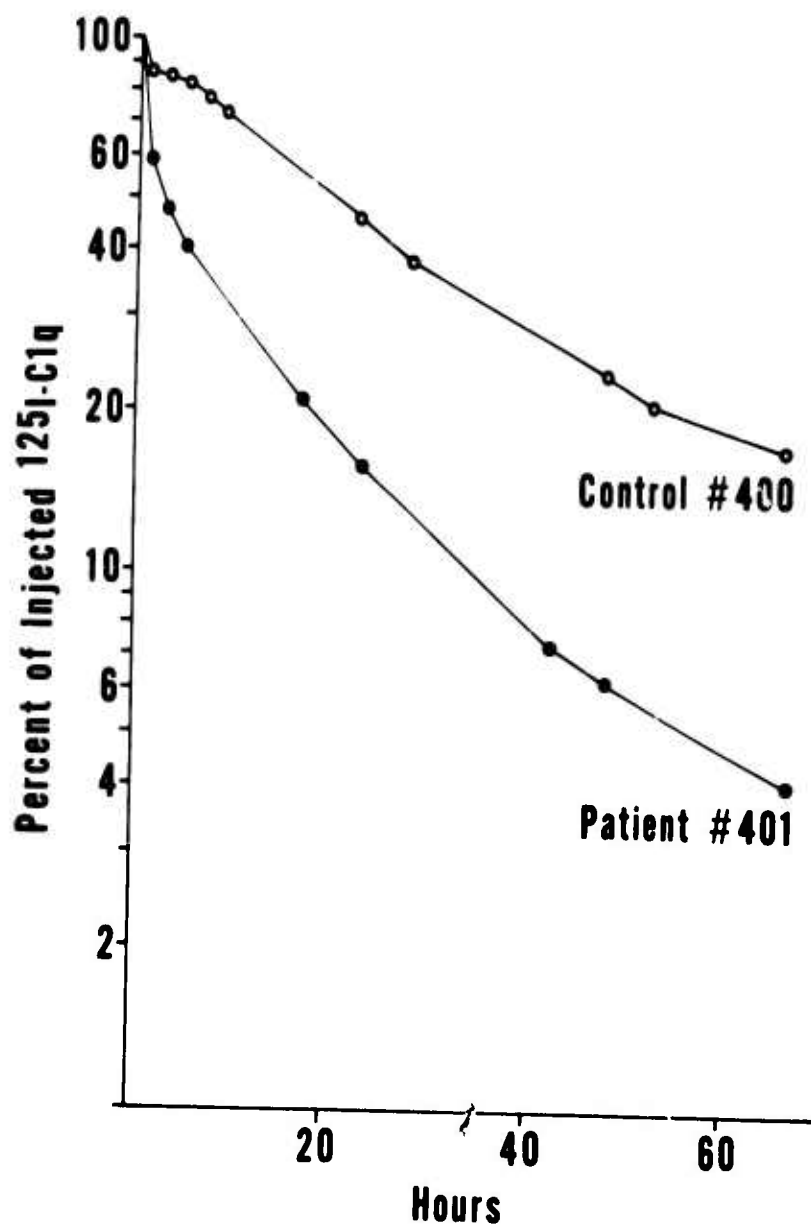


Figure 4. Corrected Elimination Rates of ^{125}I Clq in a Normal Control and DHF Patient

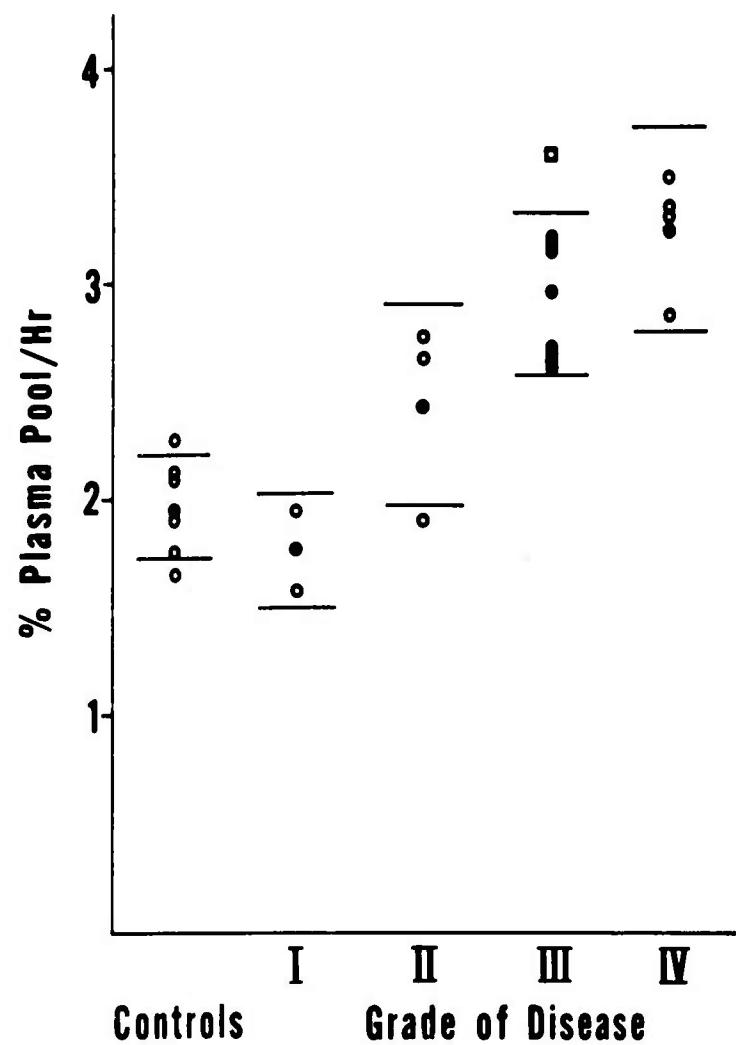


Figure 5. Corrected Elimination Rates of ^{125}I C3 in 17 DHF Patients and 6 Control Patients

● = Mean values of group
 — = \pm I.S.D of mean

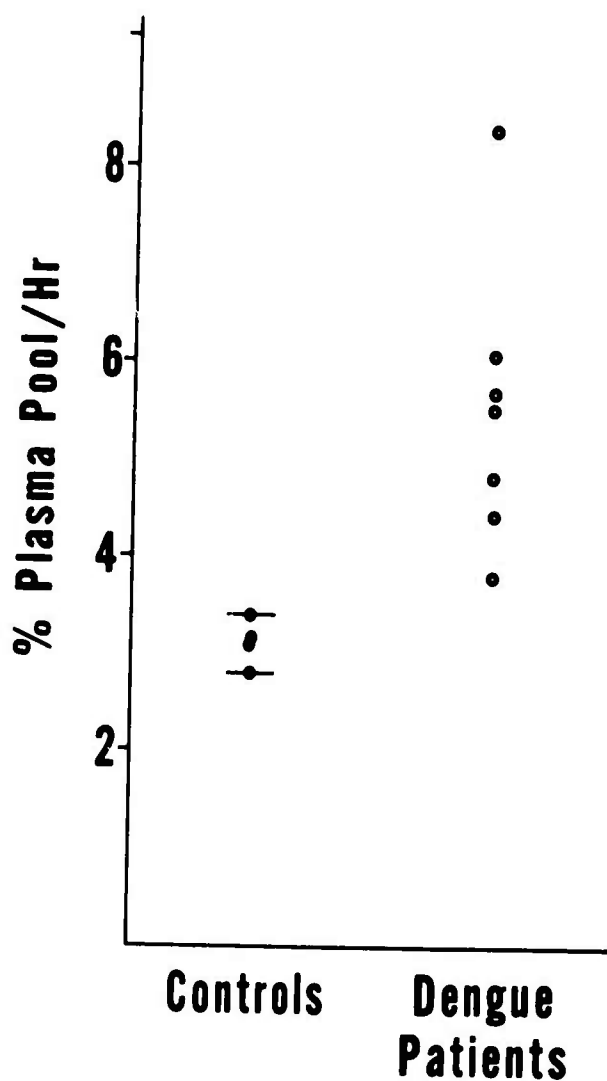


Figure 6. Corrected Elimination Rates of ^{125}I Clq in 7 DHF Patients and 3 Control Patients

○ = Mean values of group
 — = \pm I.S.D. of mean

2. Evaluation of the Plasma Kinin System in Dengue Hemorrhagic Fever

Dengue virus infections represent an important and growing public health problem in the tropics and subtropics. The pathogenesis of the severe and life-threatening manifestations found in Asia, dengue hemorrhagic fever (DHF) with and without shock, is incompletely understood. Clinical and postmortem observations suggest that pharmacologically active mediators may play a role in causing the hypovolemia and shock that occur in severe DHF. The complement system is involved and anaphylatoxins may mediate the rapid changes in vascular permeability which lead to vascular collapse.

There is circumstantial evidence that the plasma kinin system may also participate in DHF. Increased vascular permeability with hypoproteinemia and circulatory collapse are central pathophysiological events in DHF. Plasma kinins are potent endogenous mediators of increased vascular permeability and the infusion of bradykinin into man produces systemic arteriolar dilatation, decreased peripheral vascular resistance, and drop in arterial blood pressure. Bradykinin activity is elevated in early stages of endotoxin shock in monkeys. Finally the complement and fibrinolytic systems are activated in DHF, and these 2 systems are functionally interrelated with the plasma kinin system.

The development of a sensitive radioimmunoassay for bradykinin and enzymatic assays for the bradykinin activator system, which includes prekallikrein (C1 esterase) inhibitor has recently helped clarify the dynamics of the plasma kinin system by permitting simultaneous and accurate measurements of its major components. We report here sequential measurements of complement (C3) and 4 kinin system components-factor XII (Hageman), bradykinin, prekallikrein and kallikrein inhibitor-in 18 patients hospitalized with DHF, 11 of whom developed shock.

Dengue Hemorrhagic Fever Patients: All patients were admitted to the hemorrhagic fever ward of Bangkok Children's Hospital during July 1972. Seven patients were diagnosed as having DHF without shock and 11 patients as having DHF with shock (DSS) by criteria previously reported. Shock occurred on the day of admission (7 patients), hospital day 2 (2 patients), day 3 (1 patient) and day 5 (1 patient).

Fever Control Patients: Eight hospital in-patients having acute febrile illnesses not associated with dengue infection comprised the fever control (FC) group. Seven had been admitted with a presumptive diagnosis of DHF which was not tenable on the basis of their subsequent clinical course and diagnostic tests. The eighth patient developed a fever and a rash during treatment of

tuberculous meningitis.

Therapy: Dengue and FC patients received intravenous glucose-lactate-Ringer's solution for dehydration or shock, chloral hydrate for agitation, acetaminophen for hyperpyrexia, and Vitamin C. Plasma was given to 4 DSS patients and blood transfusions to one FC patient.

Non-Fever Control Subjects: Eighteen afebrile Thai children were bled once for factor XII, bradykinin, prekallikrein, and kallikrein inhibitor assays; 2 children were healthy and 16 were convalescing from surgery or acute or chronic medical conditions.

Experimental Design: Venous blood specimens from all 26 febrile patients studied were obtained on hospital days 1 (admission) 2, 4, and 6 if not previously discharged. Convalescent samples were obtained from 14 of these patients on follow-up visits 13 to 20 days after onset of their illness. All patients were bled 2 to 6 times. At least one blood specimen was drawn from most DSS patients during, or within 1-2 hours of vascular collapse; one patient was bled one day before and after the day of shock (Fig. 2).

The following laboratory tests were performed on most venous blood specimens obtained from febrile patients. Hematocrit (expressed as percent of discharge Hct), leukocyte count (per mm^3) and platelet count ($\times 10^3$ per mm^3) were done by standard methods. Serum C3 concentrations (mg/100 ml) were determined by radial immunodiffusion using agar plates commercially obtained (Hyland Laboratory, Los Angeles, Ca.); serum samples from individual patients were tested simultaneously and in duplicate. Plasma bradykinin concentrations (nanograms/ml) were determined by radioimmunoassay. The lower range of sensitivity of the radioimmunoassay ranged between .31-.67 ng/ml; measurements in this range are expressed as .67 ng/ml in subsequent analysis. The activities of plasma prekallikrein (micromoles/ml/hr of tosyl arginine methyl ester hydrolysed) and kallikrein inhibitor (inhibitor units) were determined by enzymatic assay. Plasma factor XII activity (percent of Boston standard) was determined by a modification of the activated partial thromboplastin time using congenitally deficient plasma.

The blood obtained for bradykinin, prekallikrein (PK), kallikrein inhibitor (KI) and factor XII assays was processed within 10 minutes of being drawn using plastic equipment. Blood for PK, KI and factor XII assays was added to chilled tubes containing 3.8% sodium citrate and the plasma separated and frozen at -20°C . Blood for bradykinin assay was added to tubes containing

sodium EDTA and hexadimethrine, the plasma separated, precipitated with trichloroacetic acid, and frozen. After coding, the plasma was shipped in dry ice to Boston where assays were performed on freshly thawed plasma between 2 and 5 months after the blood was collected.

Serological tests: Admission and convalescent sera were obtained from the 26 febrile patients and tested simultaneously by the hemagglutination-inhibition test in microtiter plates against 8 units of dengue serotypes 1-4 and chikungunya virus antigens. Patients were considered to have had recent dengue infections if 4-fold or greater rises in antibody titers against dengue antigens were found in paired sera, or if the anti-dengue titers were elevated and fixed ($\geq 1:640$). We did not attempt to isolate virus from these patients, but dengue 2 virus was recovered from children in Bangkok during the 1972 DHF epidemic season.

All DHF and DSS patients had serologic evidence of recent secondary dengue infections; no FC patients had evidence of recent dengue or chikungunya virus infections.

Laboratory values for the 4 groups of patients are listed in Table 1. In order to illustrate differences found between clinical groups, the admission and lowest or highest values, selected from multiple bleedings, are shown as the mean \pm S.D. The NFC values are compared with the FC admission values, and FC with DHF and DSS values; probability levels are shown (Two-tailed Student's T-test). A comparison of the FC and NFC subjects revealed significantly lower prekallikrein activity in FC patients. In contrast to fever controls, dengue patients (especially the DSS group) had greater hemoconcentration, significantly lower platelet counts, and lower C3, factor XII, and prekallikrein levels on admission and/or during the acute illness. Bradykinin and kallikrein inhibitor values were similar in all clinical groups.

The temporal profiles of platelet, C3, and kinin components in DSS patients are illustrated in Figures 1a-1f. The admission values of FC patients were selected for analysis because they are generally the lowest of repeated measurements in this group. Fig. 1a illustrates the normal C3 concentration before shock, the low levels on the day of shock, and the progressively higher concentrations during convalescence; a similar pattern exists for platelet counts (Fig. 1b). Prekallikrein (Fig. 1c) and factor XII (Fig. 1d) activities are significantly depressed before the shock day, and higher afterwards. Kallikrein inhibitor activity (Fig. 1e) shows a tendency toward low values before shock, normal levels during and for 3 days after shock, and low levels again during late

convalescence; the values are widely scattered however, and differences are not statistically significant. Bradykinin concentrations are not significantly elevated at any time in DSS patients (Fig. 1f).

Changes in laboratory values are illustrated in more detail by a patient (Fig. 2) who developed shock 3 days after admission, and therefore provided more than 1 pre-shock blood sample. The pre-shock period is characterized by a rising hematocrit and by a falling platelet count and C3 concentration. By contrast, factor XII and prekallikrein activity was low but rising when shock occurred, and normal bradykinin concentration did not change. Kallikrein inhibitor activity fluctuated widely. Similar changes were observed in another patient who developed shock 5 days after admission.

This study was designed to determine whether the plasma kinin system is activated in dengue hemorrhagic fever. In order to confirm the plausibility of bradykinin participation in DHF, we attempted to show a temporal relationship between clinical shock and elevated levels of plasma bradykinin, or of kinin system component activation. The plasma kinin system is thought to be activated sequentially as shown in Figure 3. Evidence of full activation of the kinin system should include the simultaneous demonstration of factor XII, prekallikrein, and kallikrein inhibitor depletion, and formation of bradykinin.

We found significant depression of only 2 of the 4 kinin system components - prekallikrein and factor XII. Depressed enzymatic activity of these 2 serum proteins could result from at least 3 different mechanisms: sequestration of the protein extravascularly, depressed synthesis, or an increased rate of activation and consumption. I¹²⁵ labelled albumin (mol. wt. = 49,000) crosses altered blood vessels during the shock phase of DHF, and is sequestered extravascularly.

Depressed serum concentrations of transferrin (mol. wt. = 90,000) are also found during the shock phase. It seems likely that at least part of the consistently depressed activity of prekallikrein (mol. wt. = 127,000) and Hageman factor (mol. wt. = 110,000) is due to their extravasation preceding shock. A point against extravasation, however, is that kallikrein inhibitor (MW = 50,000) was not similarly depressed before shock.

Decreased synthesis of prekallikrein and factor XII by a damaged liver may be another cause for the depressed activity of these proteins. Abnormal liver function and liver necrosis

unrelated to shock have been described in DHF. Prekallikrein levels, in particular, are a very sensitive index of liver dysfunction. Kallikrein inhibitor, although synthesized by the liver, is not very sensitive to hepatic dysfunction and remains normal in severe liver disease.

The third possibility, increased consumption of these two proteins as a consequence of their activation, is judged unlikely in view of the normal levels of kallikrein inhibitor (KI) and bradykinin found. The depletion of KI as it forms an inactive stoichiometric complex with kallikrein is a uniform finding in plasma prekallikrein activation. In DHF patients studied, there was no consistent depletion of KI at any stage of illness, suggesting that conversion of prekallikrein to kallikrein did not occur. The observed variations of KI activity during DHF could be ascribed to KI interacting with plasma enzymes other than kallikrein, such as C1 esterase, factors XI and XII, plasmin and thrombin. A second argument against consumption of prekallikrein and factor XII are the normal bradykinin concentrations found during DHF. This implies that prebradykinin was not converted to bradykinin by kallikrein, and thus that prekallikrein was not converted to kallikrein. Admittedly, if bradykinin was generated, it may have been efficiently and rapidly removed by controlling kinase systems after its formation; the half-life of bradykinin in vivo is less than 30 seconds. Alternatively, bradykinin may have been activated but operative peripherally and not detected in venous blood.

Although the foregoing evidence against prekallikrein and factor XII activation in DHF is indirect, it does suggest that if consumption does occur, pathways other than those presently considered important for bradykinin activation are operative. For example, the complement and fibrinolytic systems are activated in DHF, and factor XII may participate in this process. The low values of prekallikrein in fever control patients implies that mechanisms associated with febrile disease per se are in part responsible for the low levels in DHF; prekallikrein metabolic turnover studies should help clarify these mechanisms.

Clinical shock appeared to be more closely related temporally to changing levels of C3 and platelet counts than to levels of prekallikrein and factor XII. Two patients had lower prekallikrein and factor XII values on admission than during shock, which occurred 3 and 5 days later. If the assumption holds that the observed low levels of two of the kinin components are due to consumption, and that maximum bradykinin activation is required for the development of shock, then the time course of activation seems poorly correlated temporally to the onset of vascular collapse in these two patients.

Table 1 Comparison of Laboratory Values Obtained for Patients with Dengue Hemorrhagic Fever, Dengue Shock Syndrome and for Fever and Non-Fever Controls

Measurement ^x	Non-Fever Control (18 pts)	Fever Control (8 pts)	Dengue Hemorrhagic Fever (7 pts)	Dengue Shock Syndrome (11 pts)
Peripheral Leukocyte count: Admission	-	5900±2,700	6800±5900	8,800±5,600
Hemo - Admission concentration: Maximum	-	98±14	123±34	127±30(a)
Platelet Admission count: Lowest	-	105±3 233±80 190±89	125±33 99±73(b) 71±78(a)	137±21(b) 96±169(a) 31±11(b)
C3: Admission Lowest	-	108±31 102±28	70±13(b) 66±13(b)	86±47 61±21(b)
Factor XII: Admission Lowest	84±36 -	117±43 94±36	84±38 78±43	59±43(a) 46±30(b)
Prekallin- Admission krein: Lowest	91±16 -	74±15(c) 72±15	56±12(a) 52±15(a)	41±18(b) 37±16(b)
Kallikrein Admission Inhibitor: Lowest	0.99±0.22 -	1.00±0.25 0.75±0.24	0.92±0.17 0.70±0.11	0.85±0.37 0.64±0.30
Bradykinin: Admission Highest	1.52±1.42 -	2.39±3.55 2.71±3.50	1.52±0.68 2.41±1.42	1.06±0.70 1.9±1.67

^x all values = mean ±S.D.; units of measurement defined in text: (a) P<.05 compared to FC; (b) P<.01 compared to FC; (c) P<.05 compared to NFC; other differences not significant (P>.05).

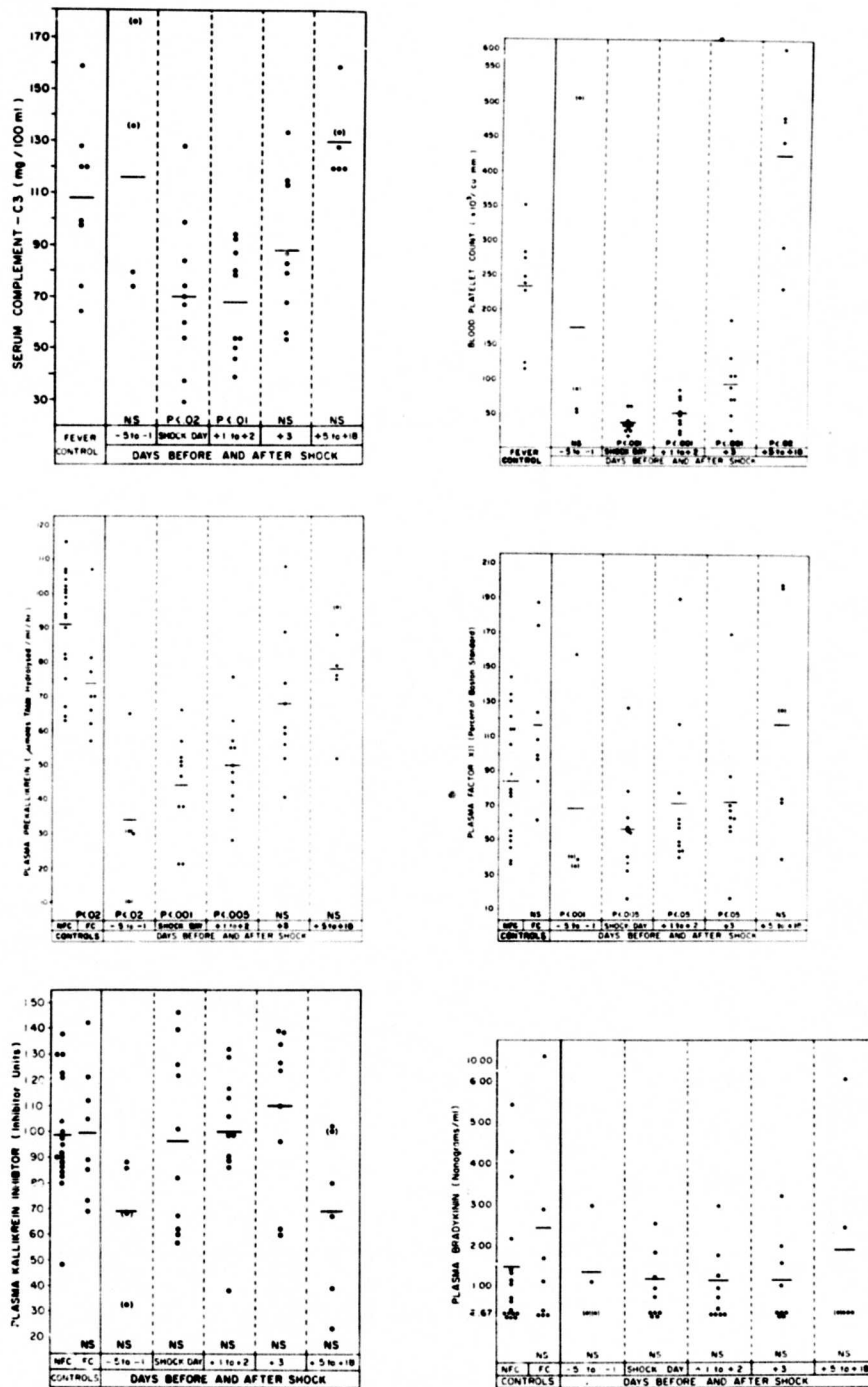


Figure 1a - 1f. Temporal profiles of C3, platelet and kinin components in patients with dengue shock syndrome (DSS). Non-fever controls (NFC) are single determinations, fever controls (FC) are admission values, and each point for DSS patients represents a single measure, except for points in parenthesis which represent the average of two or more determinations on the same individual in a given time period. Mean values are indicated by bars. Significance limits are calculated from a comparison of FC and NFC, and FC and DSS (Student's T test, two-tailed); NS = not significant ($P > .05$).

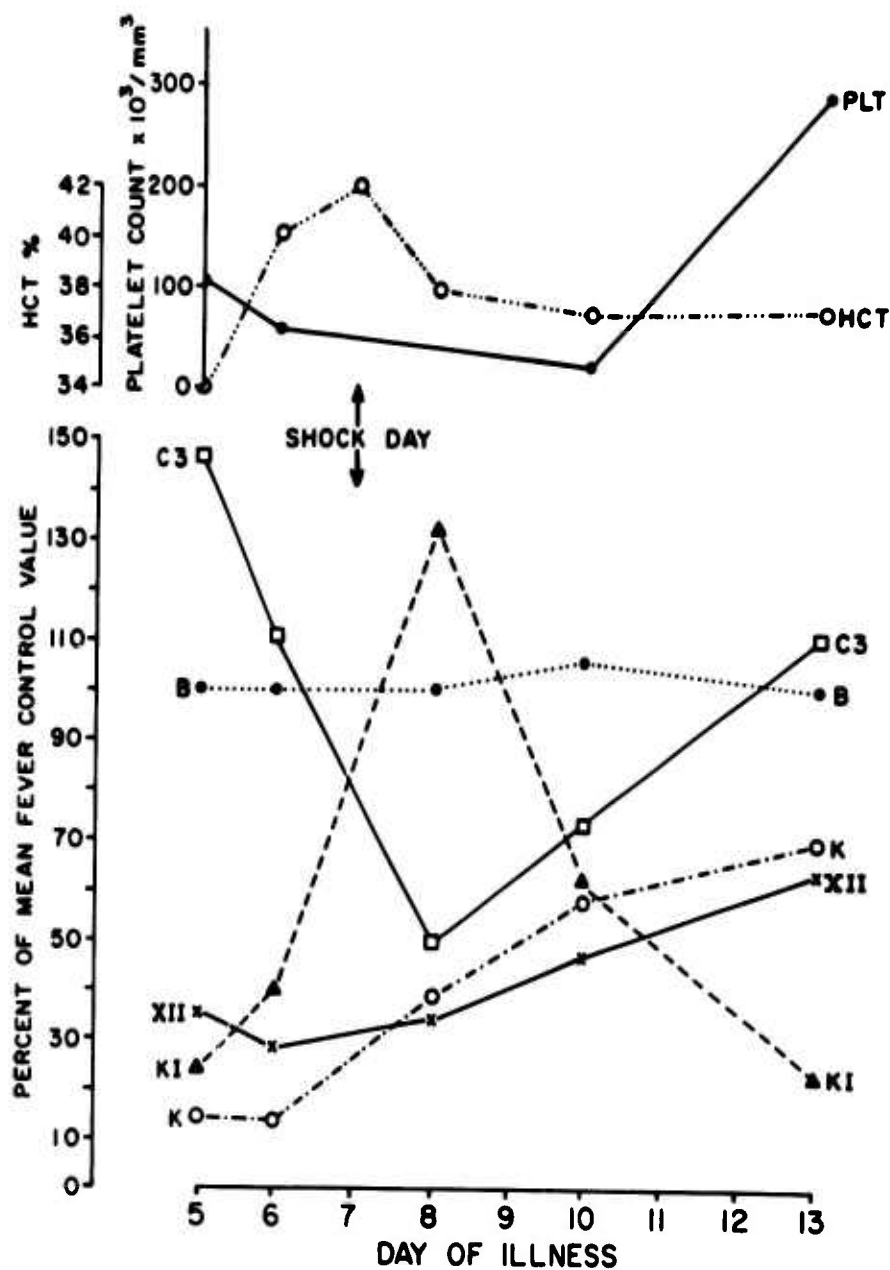


Figure 2. Changing Laboratory Values in a Patient with Dengue Shock Syndrome. PLT platelet; Hct, percent hematocrit; C3, complement; B, bradykinin; KI, kallikrein inhibitor; k, prekallikrein; XII, Factor XII.

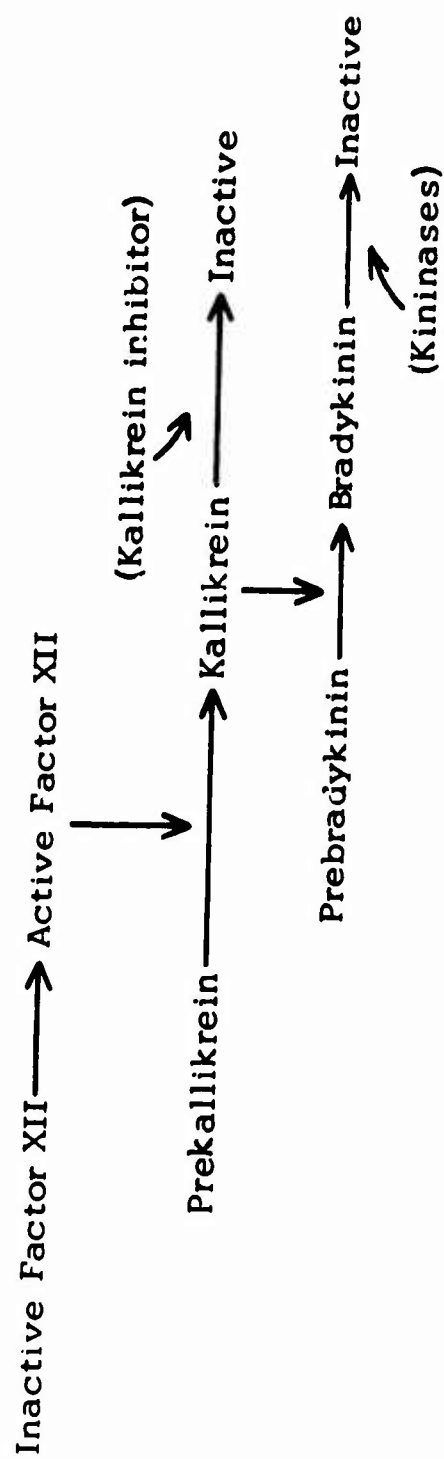


Figure 3. Pathway for Bradykinin Activation.

By contrast C3 concentrations were normal on admission in these two patients, but began to decline several days before shock and reached lowest levels 1-2 days afterwards. Platelet counts tended to parallel the changing C3 concentration. Complement (C3) activation may result in generation of the vasoactive complement peptides C3a and C5a followed by rapid removal of activated C3 from the circulation. The sequence of increased vascular permeability resulting in hypoproteinemia, hypovolemia and vascular collapse in DSS may be mediated totally or in part by vasoactive complement peptides generated prior to shock.

In order to explore the role of the plasma kinin system in the pathogenesis of dengue hemorrhagic fever (DHF), bradykinin, prekallikrein, kallikrein inhibitor, factor XII (Hageman), and serum complement (C3) were measured simultaneously during the acute and convalescent stages of illness in 7 children with DHF without shock, in 11 with dengue shock syndrome (DSS), and in 8 patients with acute febrile illnesses other than dengue (FC). Prekallikrein, factor XII and C3 levels were significantly lower in both dengue patient groups compared to FC patients, with the lowest mean levels found in DSS. However, bradykinin concentrations were not elevated and mean kallikrein inhibitor activity levels were not depressed in dengue patients. Two DSS patients studied at least 2 days before onset of shock had falling C3 levels which were more closely related temporally to the onset of shock than were their rising levels of prekallikrein. The results fail to provide convincing evidence for full activation of the plasma kinin system leading to free bradykinin or a significant role for bradykinin in the immunopathogenesis of DHF; results do re-focus attention on complement as a potentially important pharmacological mediator of dengue shock syndrome.

3. Plasma and Serum as a Source of Dengue Virus in Dengue Hemorrhagic Fever Patients

In studies reported in last year's Annual Report (pages 88-92), dengue virus was isolated from DHF patients more commonly from plasma than from serum. This difference was evident especially in children presenting with high dengue-2 HI antibody titers ($\geq 1:160$). The significance of this finding was lessened by the small numbers of patients with isolates and by technical limitations in design. Isolations from serum and from plasma were handled by different technicians.

Studies of the role of individual dengue antigens recently described by WRAIR and other laboratories in the pathogenesis of DHF are planned. Interpretation of these projected studies will

depend upon identification of the serotype of dengue virus currently infecting a DHF patient. By conventional isolation techniques using serum, isolates have been obtained from 15-20% of DHF patients and only from those patients admitted with low ($\leq 1:160$) dengue HI titers. More efficient methods of virus isolation, especially from patients presenting with high antibody titers are desired. Consequently, during the 1972 DHF season, the isolation efficiency of plasma versus serum was studied.

Studied were 68 patients admitted to Children's Hospital with a clinical diagnosis of DHF. Serum obtained on admission and discharge was tested against dengue 1-4, JEV, and Chikungunya antigens in HI tests. Patients were considered to have dengue infections if a 4-fold or greater rise in titer was shown to at least one dengue antigen or if titers were fixed but $\geq 1:640$ to at least 2 dengue antigens.

For isolation studies, 10 ml of blood was obtained on admission. A 2 ml aliquot was allowed to clot and serum was separated for isolation. The remaining 8 ml aliquot was placed into 15 ml centrifuge tubes coated with heparin (80 units); heparinized blood was centrifuged at 600 rpm for 5 minutes at 4°C. The buffy coat layer was gently aspirated. Remaining whole blood was centrifuged at 1500 rpm for 15 minutes. Plasma was aspirated and pooled with the buffy coat layer to be used as cell-rich plasma for isolation studies.

The remaining whole blood was centrifuged at 5000 rpm for 15 minutes and the upper two-thirds of the supernatant aspirated with care to avoid platelet contamination. This supernatant was recentrifuged at 5000 rpm for 15 minutes, the upper two-thirds of the supernatant was aspirated and served as cell-free plasma for isolation attempts.

Each specimen was inoculated onto 3 flasks of LLC-MK2 cells; isolation was performed by the standard method of direct and delayed plaques. All isolation attempts were performed by the same technician. White blood cell and platelet counts were carried out on all cell-rich and cell-free plasma specimens used for isolation.

Of the 68 patients studied, 13 lacked serologic evidence of dengue infection; no isolates were obtained from these patients. Of the 55 patients with serologically confirmed dengue infections, 12 dengue isolations were obtained; all were dengue 2. Table 1 shows results of isolations from serum, cell-rich plasma, and cell-free plasma from the dengue patients. Dengue 2 virus was isolated from 6 of the 7 patients with admission HI titers $\leq 1:80$ and from

Table 1. Dengue 2 Isolations from Serum, Cell-rich Plasma, and Cell-free Plasma Related to Dengue 2 HI Titer in Serum Used for Isolation

Dengue 2 HI Titer (Reciprocal)	Number of Patients	Number of Isolates			
		Total	Serum	Plasma Cell-rich	Cell-free
≤ 20	3	3	2	3	3
40	3	3	3	2	2
80	1	0			
160	6	1	1	1	1
320	5	1	0	1	1
640	16	2	0	1	2
1280	12	2	0	2	2
2560	4	0			
5120	2	0			
10240	0	0			
≥ 20480	3	0			
Total	55	12	6	10	11

7 of the 13 patients studied with HI titers $\leq 1:160$. In these patients, isolation was equally efficient from serum or plasma specimens.

Dengue 2 virus was isolated also from 5 of 33 patients admitted with dengue 2 HI titers of 1:320-1:1280. No isolates were obtained from serum of these patients, but 5 isolates were from cell-free and 4 from cell-rich plasma.

No isolates were obtained from the 9 patients with initial dengue 2 HI titers $\geq 1:2560$.

Little difference in isolation efficiency between cell-rich and cell-free plasma was found, even in those patients without virus in serum. Generally, these cell-free plasma preparations were indeed cell-free. White blood cells were not detected in any cell-free plasma preparation yielding virus, while platelet contamination ($1-3 \times 10^3/\text{mm}^3$) was detected in only 5 of 12 cell-free preparations yielding virus. In the 5 patients with serum negative for dengue virus, WBC and platelets were not detected in cell-free plasma yielding virus in 3 patients.

These results confirm the limited data from the 1972 study in defining a small but important difference in the efficiency of isolation of dengue virus from plasma and serum of DHF patients. No difference in isolation efficiency was found in patients with low ($\leq 1:160$) admission dengue 2 HI titers, but all 5 isolates from patients with initial titers $\geq 1:320$ were obtained from plasma only.

Our initial hypothesis that an increased isolation rate from plasma might be due to the presence of cell or platelet associated virus in plasma is not supported since no differences between cell-rich and cell-free plasma was found in patients lacking virus in serum. That the decrease in isolation efficiency might be due to adherence of virus to fibrin in the clot does not seem likely in view of the differences between isolation from serum in patients with low and high admission HI titers. Possibly, heparin itself influences neutralization of virus in high-titered plasma, permitting incompletely neutralized virus to infect LLC-MK2 cells. Further studies will be required to test this hypothesis. Since virus was isolated from only 22% of the patients with DHF by the most efficient specimen (plasma), other approaches to increase the efficiency of dengue isolation in DHF patients are indicated.

4. Efficiency of Peripheral Blood Collected on Filter Paper Discs and One Dengue Antigen in Serologic Diagnosis of Dengue Hemorrhagic Fever.

For routine serologic diagnosis of dengue infections in this laboratory, serum has been tested against antigens of all four dengue serotypes. This traditional approach has two significant disadvantages in a large scale surveillance program for DHF. First, obtaining blood by venipuncture, aseptic separation of serum and transportation of serum to a central laboratory requires manpower, equipment, and logistical support not often available to smaller provincial hospitals. Second, testing sera against four viral antigens is expensive (cost, \$2.00 US per test) and time-consuming, limiting the number of patients which can be tested serologically.

For serologic tests against viruses other than dengue (rubella, coronaviruses), peripheral blood collected on a filter paper disc has proven a practical and economical collection system in extensive field research projects. The purpose of the study reported was to compare filter paper disc specimens with serum specimens for serologic diagnosis of dengue in patients with suspected DHF. A second goal was to compare the efficiency of the HI test in serologic diagnosis of dengue using only one dengue antigen versus all four dengue antigens. This research has important implications in serologic diagnosis of dengue infections under adverse military field conditions.

Sixty-four patients admitted to Children's Hospital with a diagnosis of DHF were studied. Dengue viruses were isolated from 11 of these patients; all isolates were dengue 2. Acute serum drawn on admission and convalescent serum drawn on discharge on each patient were tested for antibody to the four dengue serotypes by the standard method of Clarke and Casals modified to microtiter; 8-16 units of antigens were used. In addition, peripheral blood obtained by finger prick was collected on two filter paper discs (Schleider and Schuell, No. 740-E, diameter 12.7 mm). One set of acute and convalescent discs from each patient was stored at 4°C and the other at 27°C for one week (to mimic the effect of ambient temperature). Both sets of discs were soaked with 0.4 ml borate saline (pH 9.0) and incubated at 4°C overnight. One half ml of a 25% acid-washed kaolin solution in borate saline was added and incubated at room temperature for 20 minutes with occasional agitation. The eluate was separated by centrifugation at 2500 rpm for 30 minutes, treated with 0.02 ml of packed goose RBC at 4°C for 30 minutes with occasional agitation and then centrifuged. Since the IgG concentrations of several eluates tested approximated 5% of that of companion sera, the final dilution of the eluate obtained was considered to be a 1:20 dilution of whole serum. The eluate was tested for HI antibodies to dengue 2 and dengue 4 antigens by the same technique used for serum specimens.

Shown in Table 1 are the differences in HI titer (log 2) between the filter paper technique and serum for 128 individual

specimens tested. There is close concordance in the two techniques for dengue 2 HI antibody titers; 36% of the filter paper titers were within one dilution of the serum titer. A different pattern was seen with dengue 4 antigen. Only 61% of filter paper titers were within one dilution of the serum titer; the distribution of filter paper titers was skewed considerably toward lower titers than were obtained in sera. Technical reasons for the discrepancy with the two antigens are not apparent; indeed, identical aliquots of the eluate of each specimen were tested with both antigens simultaneously.

A comparison of titers obtained from filter paper discs stored at 4°C and 37°C for one week against the two dengue antigens is shown in Table 2. Tested were 52 sera from 26 DHF patients. Storage at 37°C did not change HI titers significantly in comparison with storage at 4°C.

Table 3 compares diagnosis of dengue infections using dengue 2 antigen alone by serum and by filter paper disc specimens in the 64 patients studied. Rises in titer were found in 32 patients using serum compared with 29 using discs. Concordance of the two methods was found in 20 patients with titer rises, in 10 with high fixed titers, and in 13 patients with low fixed titers. Discrepancies were limited to variations between titer rises and high fixed titers by both techniques. The results with dengue 4 antigen were similar (Table 4).

The efficiency of serologic diagnosis of dengue infections using the filter disc collection method and one dengue antigen in comparison with serum and four dengue antigens is shown in Table 5 (dengue 2 antigen) and Table 6 (dengue 4 antigen). In this analysis a 4-fold or greater titer rise to any one of the four dengue antigens in serum specimens was considered indicative of dengue infection. Results were similar with both antigens; conventional serologic techniques identified 46 dengue infections by rise in HI titer in the 64 patients studied while filter paper discs detected infections in 29 (D2) and 30 (D4) patients. All infected patients not detected by the filter paper technique had high fixed titers ($\geq 1:640$) to the dengue antigen used. Both methods were concordant in patients with low fixed titers (without evidence of dengue infection). If a rise to either D2 or D4 was used to indicate dengue infection, infections were identified in 31 of the 46 patients who had a serum titer rise to one of the four dengue antigens.

Little difference in the efficiency of serologic diagnosis of dengue infections with 64 DHF patients studied was found between the peripheral blood filter paper disc specimens and serum

specimens when tested with one dengue antigen. Storage of discs at 37°C for one week did not change dengue HI titers. The data suggested that the more convenient filter paper disc can be used for dengue diagnosis with little decrease in diagnostic efficiency.

Considerable decrease in efficiency of dengue serologic diagnosis accrued when one dengue antigen was used instead of all four dengue serotypes. Using serum specimens, only 33 patients (D2 antigen) and 30 patients (D4 antigen) had diagnostic titer rises while 46 patients showed rises when all four dengue antigens were used. By filter paper using one dengue antigen, 29 (D2 antigen) and 30 (D4 antigen) showed rises. All patients with rises detected by four antigens but no rise with single antigens (D2 or D4) had fixed titers of $\geq 1:640$ against the single antigens. If high fixed titers ($\geq 1:640$) (in addition to four-fold titer rises) are accepted as serologic criteria of recent dengue infections, the use of one dengue antigen results in little decrease in diagnostic efficiency. Patients without serologic evidence of dengue by conventional techniques were correctly identified by the filter paper disc-single antigen test. Although the lack of concordance in titers to dengue 4 antigen between the two tests suggests caution in the application of the filter disc technique for antibody prevalence data, the results suggest that it is an acceptable technique for confirmation of dengue infections in a DHF surveillance program.

Table 1

Concordance between HI Titers obtained by filter-paper technique and simultaneous serum in acute and convalescent specimens from 64 patients.

Difference in HI Titer (Log 2) of filter-paper method from serum	HA Antigens	
	Dengue 2 (no. sera)	Dengue 4 (no. sera)
-6	0	2
-5	0	1
-4	2	6
-3	1	18
-2	9	21
-1	23	26
0	62	39
+1	25	13
+2	6	2
+3	0	0

Table 2
Comparison of HI titers from eluates of filter paper discs stored at
37°C and 4°C (26 patients).

Difference in HI titer (Log 2) of discs stored at 37°C and 4°C	HA Antigens	
	Dengue 2 (no. sera)	Dengue 4 (no. sera)
-3	0	0
-2	3	2
-1	14	14
0	31	30
+1	3	4
+2	1	2
+3	0	0

Table 3

Efficiency of Serologic Diagnosis of Dengue Infection by Filter Paper Discs Compared with Serum (Dengue 2 HA)

Filter Paper Disc		Serum			
		Titer rise	No Titer rise		Totals
			≥ 1:640 Titer (no. patients)	<1:640 Titer (no. patients)	
Titer rise		20	8	1	29
No Titer rise	≥ 1:640	11	10	0	21
	<1:640	1	0	13	14
Totals		32	18	14	64

Table 4

Efficiency of Serologic Diagnosis of Dengue Infection by Filter Paper Discs Compared with Serum (Dengue 4 HA)

Filter Paper Disc		Serum			
		Titer rise	No Titer rise		Totals
			<u>≥</u> 1:640 Titer (no. patients)	<u><</u> 1:640 Titer (no. patients)	
Titer rise		21	8	0	29
No Titer rise	<u>≥</u> 1:640	7	15	0	22
	<u><</u> 1:640	0	0	13	13
Totals		28	23	13	64

Table 5

Efficiency of Serologic Diagnosis by Filter Paper Discs; Discs and
D₂ HA Compared to Serum and D₁ - D₄ HA

Filter Paper Disc D ₂ Antigen		Serum, 4 Dengue Antigens			
		Titer rise	No Titer rise		Totals
			≥1:640 Titer (no. patients)	<1:640 Titer (no. patients)	
Titer rise		28	1	0	29
No Titer rise	≥1:640	17	4	0	21
	<1:640	1	0	13	14
Totals		46	5	13	64

Table 6

Efficiency of Serologic Diagnosis by Filter Paper Disc; Discs and
D₁ HA Compared to Serum and D₁ - D₄ HA

Filter Paper Disc D ₄ Antigen		Serum, 4 Dengue Antigens			
		Titer rise	No Titer rise		Totals
			≥1:640 Titer (no. patients)	<1:640 Titer (no. patients)	
Titer rise		29	1	0	30
No Titer rise	≥1:640	17	4	0	21
	<1:640	0	0	13	13
Totals		46	5	13	64

B. JAPANESE ENCEPHALITIS

1. Human Immunoglobulin M Antibody in the Serodiagnosis of Japanese Encephalitis Virus Infections.

The serological identification of a primary group B arbovirus infection can usually be made on the basis of a monospecific antibody response to a type specific virus antigen, measured by the standard serological tests of HI, CF, or Nt. However, the high-titered heterospecific antibody found in serum after sequential infections with group B arboviruses hinders specific identification of the most recent infection. In addition, the rapid anamnestic antibody titer rise in secondary group B infections, together with delays in obtaining the acute phase serum specimens from some patients, often makes it impossible to detect a titer rise in whole serum.

Serum immunoglobulin M (IgM) has been found to be more capable than serum immunoglobulin G (IgG) of distinguishing antigenic differences between certain virus types within non-arbovirus groups (2, 3, 4, 5). Similarly IgM antibody produced in response to group B arbovirus infections of rabbits and guinea pigs was more specific than IgG for the infecting virus, and IgM antibody reacting monospecifically with JEV antigen provided a specific serological diagnosis for first JEV infection in gibbons previously sensitized with dengue. The usefulness of the IgM antibody assay in human dengue infections has recently been reported.

We have encountered many patients in Southeast Asia who have either primary infections with monospecific fixed or falling titers to JEV or dengue, or secondary infections with cross-reactive titers to these viruses. In an attempt to improve the efficiency of serodiagnosis of these infections, we have compared the immunospecificity of isolated IgM HI antibody with the specificity of HI, CF and Nt antibody in whole serum obtained from groups of well-studied patients. This paper describes our experience using IgM antibody analysis in the serodiagnosis of 88 patients hospitalized in Vietnam and Thailand with presumed Japanese encephalitis. We also present the results of IgM antibody titrations on the serum of Thai persons with clinically inapparent JEV infections.

Patients with Japanese Encephalitis: Acute and convalescent bloods were collected from 2 groups of patients hospitalized with fever and acute encephalitis. The first group consisted of 23 American military personnel, between 18 and 27 yrs of age, hospitalized at the 93rd Evacuation Hospital, Long Binh, Vietnam,

from May to October 1970. The presumptive diagnosis of encephalitis was made on the basis of the clinical triad of headache, fever, and central nervous signs and symptoms associated with an abnormal cerebrospinal fluid (CSF), which was sterile for bacteria on culture. A CSF was considered to be abnormal if greater than 10 white cells/cmm were found, or if the protein was elevated above 45 mg%. Convalescent phase sera for arbovirus serology were drawn from each patient 10 to 14 days after the acute phase sera. The time between the onset of illness and the acute phase serum sample ranged from 2 to 17 days. Although 3 patients died, no postmortem brain specimens were obtained for viral isolation attempts; JEV was isolated, however, from the brain of an American soldier dying with encephalitis during the 1969 epidemic of JE which occurred in the Saigon-Long Binh area of Vietnam. All patients had presumably been immunized with 17-D yellow fever virus vaccine prior to their arrival in Vietnam; no patients gave a past history compatible with clinical dengue fever.

Serum pairs from the second group of encephalitis patients, 65 in number, were selected from a serum bank representing more than 120 Thai patients. The patients were hospitalized during a 1970 JE epidemic which occurred in the Chiangmai and Lampang Valleys of Northern Thailand. The presumptive diagnosis of JE was made using clinical criteria described above. The age of these 64 Thai patients ranged from 3 to 47 yrs, and approximately 2/3 were males. JEV was isolated in this laboratory from the brain of a fatal case, and 13 more isolates of JEV were recovered from vector mosquito pools collected in Chiangmai during the epidemic. Detailed epidemiological and clinical descriptions of these and additional patients hospitalized during the 1970 epidemic will be published elsewhere. Acute phase serum specimens were drawn 1-24 days after onset of disease, with a median of 4 days. Convalescent phase sera were drawn from each patient 3 to 72 days after the acute specimen, with a median interval of 14 days.

Subjects with Inapparent Infections: Sera obtained from two groups of healthy Thai persons residing in Chiangmai Valley were tested for evidence of recent JEV or group B infection. The first group consisted of more than 70 family members of hospitalized JE patients. These family members volunteered for serial bleedings performed prospectively over 2 to 8 week intervals during the 1970 epidemic. Serum from eleven family members with evidence of inapparent infections were selected for IgM analysis. Another group of 31 subjects was selected from over 400 Chiangmai Valley villagers and Chiangmai city schoolchildren who participated in a study designed to monitor the incidence of inapparent JEV infection in Chiangmai Valley in 1970. They were bled at approximately 3 month intervals. Persons in Chiangmai Valley reside in an area where JEV, dengue and Tembusu viruses are endemic.

Serological tests: HI and CF tests were performed in microtiter plates as described previously, using 8 units of HA arbovirus antigens for the HI test, and 8 CF units of antigen and 2 units of complement for the CF test. The Nt test previously described was employed with minor modifications, in that plaques in JEV and dengue infected cultures were counted on days 5 and 6, respectively.

The following virus strains were used for the 3 serological tests: JEV (Nakayama), dengue 1 (Hawaii), dengue 2 (New Guinea "C"), dengue 3 (H-87), and dengue 4 (H-241). In addition, Tembusu virus (LGLT-377) and Wesselsbron virus (BKM 367-66) were used in HI tests of sera from 11 individuals with inapparent infections.

Treatment of Whole Serum with 2-mercaptoethanol: One part of a 1:10 dilution of 2-ME in borate saline (0.2 molar), pH 9.0, was added to 9 parts of goose-erythrocyte adsorbed, acetone-treated serum. The mixture was incubated at 37°C for 30 minutes and then placed at 4°C for 30 minutes. The 2-ME treated and untreated aliquots of the same serum were then diluted simultaneously for the HI test run against 8-16 HA units of D 1-4 and JEV antigens. If the HI antibody titer after treatment was reduced to or below one-fourth of the titer before treatment, the serum was judged to contain 2-ME sensitive (IgM) antibody.

Serum Fractionation by Sucrose Density Gradient Centrifugation: The fractionation of serum followed standard SDGC methodology. Briefly, 0.125 ml of heat-inactivated serum was diluted with normal saline to 0.25 ml, adsorbed with goose erythrocytes, and then layered on a 10% to 40% sucrose gradient (5.5 ml total volume). Following centrifugation at 35,000 rpm for 18 hours, 12 fractions of the gradient were collected dropwise through a pin-hole drilled in the bottom of the centrifuge tube. Fractions 1 thru 7 were collected in 0.3 ml aliquots, while fractions 8 thru 12 were collected in 0.5 ml volumes. Each fraction was divided into 2 aliquots. One aliquot was treated with 2-ME (0.135 ml sucrose fraction plus 0.15 ml of 0.2 molar 2-ME) for 30 min at 37°C and then for 30 min at 4°C; the second aliquot was treated with 0.15 ml buffer similarly. The untreated (control) and 2-ME-treated aliquots were then diluted for HI titration against 8-16 units of HA arbovirus antigens. Serum from patients with encephalitis were routinely tested against JEV & D1-4 antigens; healthy persons were tested against JEV, one or more dengue serotypes, Wesselsbron, and Tembusu antigens. The concentration of IgM and IgG in the 12 serum-sucrose fractions of each of 12 sera was determined by radial immunodiffusion in agar

("Immunoplates" Hyland Laboratories, Los Angeles, Calif). All of the IgM detectable by radial immunodiffusion was concentrated into serum-sucrose fractions 3, 4 & 5, and occasionally into fractions 2 thru 6. Between 80-100% of the detectable IgG was found in fractions 7-12, while the remainder, if any, was detected in fractions 3-6, and accounted for 0%-40% of the immunoglobulins contained in these early fractions. Because IgM was reproducibly concentrated into 3 fractions (3, 4, 5), these were always selected without pooling for 2-ME treatment and HI titrations; the remaining 9 fractions were discarded. As previously reported the non-specific HA inhibitors were isolated in the top of the sucrose gradient (fraction-12); therefore sera were not extracted with acetone before HI testing.

Serological Criteria; Whole Serum: Encephalitis patients were divided into 2 serological groups on the basis of their whole serum HI & CF antibody responses to JEV and D1-4 antigens. The first group had titer rises (≥ 4 -fold) to JEV alone, or to JEV & D4 with higher JEV convalescent titers. These patients were presumed to have had a recent primary immune response to their first exposure to a group B arbovirus (JEV). The second group were considered to have had a probable recent JEV infection secondary to prior group B arbovirus experience. This secondary-type serological pattern was characterized by an anamnestic antibody response, with high convalescent titers (generally $\geq 1:640$ by HI and $\geq 1:64$ by CF) to JEV and to 2 or more dengue serotypes; the JEV convalescent titer was usually ≥ 4 -fold higher than any one of the 4 dengue serotypes. Serological pattern varied according to the test employed. The one test providing the most definitive serological result, i.e., monospecific instead of heterospecific, and rising rather than fixed or falling titers, was recorded. Patients with monospecific fixed or falling titers against JEV in paired serum spaced greater than 7 days apart were considered to have been infected once with JEV at some unspecified time in the past. Patients with heterospecific fixed or falling titers to JEV and to two or more dengue serotypes were considered to have been infected more than once by group B arboviruses in the past. Representative primary and secondary serological patterns (Table 1) illustrate that any one of the three serological tests provided a specific diagnosis of a recent or remote JEV primary infection, whereas none of the 3 serological tests identified the type-specific group B arbovirus in secondary infections.

Treatment of Whole Serum with 2-mercaptoethanol: In an attempt to confirm JEV as the Specific group B arbovirus responsible for acute encephalitis in patients with secondary infections, we tested for the presence and reactivity of IgM antibody by treating whole

serum with 2-ME. 2-ME labile antibody, reactive against JEV but not D1-4, was detected in the sera of 6 of 7 Thai and American patients with primary JEV infections, but in none of 13 patients with secondary infections. These results indicate that IgM antibody does appear in the majority of patients with a primary immune response to a recent JEV infection, and that the antibody is specific for JEV. The inability to detect IgM antibody after secondary infections may have been the consequence of high-titered IgG antibody masking lower-titered IgM antibody in whole serum. We therefore isolated IgM and IgG from whole serum by sucrose density gradient centrifugation (SDGC).

Serological Criteria: SDGC Fractions of Whole Serum: Virus specific IgM antibody was considered present in serum-sucrose fractions of whole serum when the HI titer against one of the HA antigens fell after 2-ME treatment. The fall was considered significant when 2-ME reduced the HI titer to $<1/8$ of the control titer in any one of the 3 IgM-rich fractions, or to $<1/4$ of the control in 2 or more fractions. These criteria are weighted in favor of eliminating false positive IgM results. In a small number of sera, we noted a 4-fold HI titer drop in one IgM-rich fraction and a 2-fold titer reduction in 2 additional IgM fractions after 2-ME treatment. Such sera were considered to contain "trace" IgM antibody activity against the antigen(s) in question. The IgM antibody titer was considered to rise or fall significantly when the titer between acute and convalescent sera differed ≥ 2 -fold in at least 2 fractions, or ≥ 4 -fold in at least one fraction. Low-titered IgM antibody was lost after the unavoidable 10-fold dilution of serum in the sucrose gradient. This dilutional loss of IgM antibody activity, resulting in false negative results, was noted particularly in serum having JEV HI titers of $<1:40$.

Repeated testing of IgM antibody negative and positive serum (15 sera refractionated and titered 2 to 6 times) resulted in reproducible titrations in greater than 95% of runs. The SDGC procedure was precise in that doubling the serum volume placed on the gradient resulted in a 2-fold increase of IgM and IgG antibody titers. Storage at -20°C for 12 months, and as many as 4 freeze-thaw cycles, produced no significant loss of JEV IgM antibody activity.

Serum IgM Antibody After Japanese Encephalitis: The paired sera from the 4 patients shown in Table 1 were fractionated and the results are listed in Table 2. The 2-ME labile HI antibody limited to fractions 3-5, which contained all immunoprecipitable IgM, reacted with JEV but not with D 1-4. In contrast, HI antibody in fractions 7-12 (not shown) reacted in high titers (1:16 to $>1:128$) with both JEV and dengue antigens, was 2-ME resistant, and was

IgG by radial immunodiffusion. We conclude that the SDGC procedure partially or completely isolates IgM from IgG and thereby permits measurement of the IgM antibody titer. Furthermore, the results in Table 2 indicate that JEV-specific IgM antibody is produced not only in primary JEV infections, but after JEV infections in patients with previous group B arbovirus experience as well. The IgM antibody titers in paired sera may be rising (Patients A and C), fixed (not illustrated), or falling (Patients B and D). The contamination of IgM by IgG probably accounts for the 2-ME resistant antibody found in some IgM-rich fractions. This contamination was observed more often in high-titered sera (Patients C and D) than in low-titered sera (Patients A and B). The presence of serum IgM immunoreactive against JEV in these patients with acute encephalitis provides additional serological confirmation of a recent JEV infection.

A further comparison of whole serum and IgM antibody patterns in 23 American and 65 Thai encephalitis patients is presented in Table 3. Twenty-one American troops had IgM antibody reactive only against JEV, and 19 of these 21 had rising IgM titers. One patient (E, Table 4) had rising IgM convalescent antibody titers cross-reacting with JEV ($>1:128$) and D4(1:8). IgM antibody was not detected in a final patient, a female nurse stationed in Saigon, who had low-fixed whole serum HI titers of 1:20-1:40 against JEV and D4. Thus rising titers in conventional serological tests specifically confirmed a recent JEV infection in only 7 American patients (30%). HI titration of isolated IgM confirmed the diagnosis in 19 patients (83%), and led to a presumptive diagnosis of JEV in 3 more persons with fixed, falling or weakly heterospecific IgM titers.

JEV IgM antibody was found in 41 of 65 hospitalized Thai children and adults (Table 3). Thirty-nine of these 41 persons had JEV monospecific activity, and 31 of these 39 showed rising IgM titers; the remaining 8 patients had fixed or falling IgM titers. Most IgM negative patients fell into the secondary infection group; possible reasons for this disparate distribution of negative patients will be discussed later. Two of the 41 Thai patients displayed cross-reactive IgM antibody titers (Table 4). The high JEV and low D4 IgM titers in patient F suggest a recent JEV infection. In patient G, the unusual combination of high-titered, cross-reactive IgM against JEV and D1, 2 and 3 precludes the presumptive serodiagnosis of JE.

As seen in Table 3, rising titers in conventional serological tests in Thai patients specifically confirmed a recent JEV infection in 19 persons (29%), while the diagnosis was confirmed by JEV-specific, rising IgM antibody titers in 31 persons (48%). In

addition, the JEV IgM titers were fixed in 1 patient, falling in 7, and weakly heterospecific in 1 (patient F; Table 4), providing a presumptive or confirmed diagnosis of JEV in 40 cases (61%).

IgM antibody reacted monospecifically with JEV in 60 of 63 Thai & American patients whose sera contained IgM antibody activity.

The diagnostic efficacy of each of the 4 serological tests used in this study is compared in Table 5. Only patients with monospecific, rising titers to JEV are considered positive. Twice as many presumed cases of JE were confirmed as recent JEV infections by IgM analysis than by any other single serological test. The apparent superiority of IgM analysis could be increased further if the 12 patients with fixed, falling or low titered hetero-specific IgM patterns are included.

Serum IgM Antibody After Inapparent JEV Infections: It was of interest to determine whether serum obtained from persons with inapparent JEV and secondary group B arbovirus infections contained JEV-specific IgM antibody. Thirty-six sera from 31 apparently healthy Chiangmai villagers and urban school children, with evidence of subclinical infections detected serologically at 12-16 week interval bleedings, were fractionated by SDGC (Table 6). Only one serum from a Chiangmai city school child contained trace amounts of antibody to D2. The 35 remaining sera contained no detectable IgM antibody. These results raised the possibility that IgM antibody may not be produced after inapparent JEV infections.

In order to further investigate this possibility, we fractionated sera obtained from 11 additional Chiangmai villagers with inapparent JEV or group B infections. These infections were diagnosed by antibody titer rises in serial sera drawn prospectively at 2 to 8 week intervals. Five of the 11 subjects had primary rising titers; 4 of these produced IgM antibody reactive with JEV but not D1-4, Tembusu, or Wesselsbron viruses (Table 6). Virus-specific IgM antibody is therefore produced in inapparent JEV infections. In contrast to the findings in encephalitis patients, no IgM antibody activity was detected in 6 serum pairs showing primary falling or secondary infection patterns.

The above studies may be summarized as follows: Paired sera, obtained from 88 American and Thai patients hospitalized with presumed Japanese encephalitis, were tested by the standard serological techniques of hemagglutination inhibition (HI), complement fixation, and plaque reduction neutralization. On the basis of these tests, 35 patients had recent or remote primary JEV infections, while 53 patients had secondary group B arbovirus

Table 1. Representative serological patterns in whole sera from patients with fever and encephalitis.

Patient	Day of Disease	Reciprocal serum antibody titer																Sero _{xxx} Pattern
		HI						CF						Nf				
		JEV	D4	D3	D2	D1	JEV	D4	D3	D2	D1	JEV	D4	D3	D2	D1		
A 18 yr male American	6	40	0 _{xxx}	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1 ^{Or}
	12	320	40	20	20	20	32	0	0	0	0	250	0	0	0	0		
B 9 yr male Thai	4	320	0	0	0	0	0	0	0	0	0	NOT DONE						1 ^{Or} x
	16	320	20	0	0	0	4	0	0	0								
C 11 yr female Thai	4	320	640	320	160	320	8	16	16	16	16	40	40	300	640	550	2 ^{Or}	
	13	2560	1280	1280	320	640	64	64	64	64	64	320	100	640	2200	2000		
D 16 yr male Thai	24	5120	1280	2560	640	5120	128	128	32	64	64	350	2300	40	800	1200	2 ^{Or} x	
	35	2560	1280	2560	1280	2560	16	128	32	32	64	270	900	40	800	2560		

xx For this and all subsequent tables: 1⁰ = primary JEV infection,
2⁰ = secondary group B arbovirus infection, R = rising titer (5-4-fold),
F_x = fixed titer, F₁ = falling titer (5-4-fold) by HI or CF.

xxx For this and all subsequent tables: 0 = <1:20 for HI, <1:4 for CF, <1:10 for Nt.

Table 2. IgM antibody titration of paired sera from encephalitis patients with primary JEV & secondary group B arbovirus infections.

Pt Whole ^{xx} Serum Serological Pattern	Day of Disease	Serum [‡] Sucrose Fraction	Reciprocal HI antibody titer									
			JEV		D4		D3		D2		D1	
			C	2-ME ^{**}	C	2-ME	C	2-ME	C	2-ME	C	2-ME
A 1 ^{OR}	6	3	2	0	0	0	0	0	0	0	0	0
		4	2	0	0	0	0	0	0	0	0	0
		5	2	2	0	0	0	0	0	0	0	0
	12	3	8	0	0	0	0	0	0	0	0	0
		4	16	0	0	0	0	0	0	0	0	0
		5	64	0	0	0	0	0	0	0	0	0
B 1 ^{OF_x}	4	3	8	2	2	2	2	2	0	2	2	2
		4	16	0	2	0	2	2	2	2	2	2
		5	16	2	2	0	4	4	2	2	4	4
	16	3	8	0	2	0	2	2	2	0	2	0
		4	16	2	2	0	2	2	2	0	2	0
		5	4	2	0	0	4	8	2	0	2	2
C 2 ^{OR}	4	3	2	2	2	4	4	4	2	4	2	4
		4	4	2	2	4	4	4	4	4	4	4
		5	4	2	2	4	8	4	4	4	4	4
	13	3	32	8	4	4	4	4	4	4	8	8
		4	64	8	4	4	4	4	4	4	8	8
		5	32	8	4	4	4	4	4	4	8	8
D 2 ^{OF_x}	24	3	32	8	8	8	16	16	8	8	8	8
		4	32	8	8	8	16	8	8	8	8	8
		5	32	8	8	4	16	8	8	8	16	8
	35	3	16	8	8	8	16	16	8	16	8	8
		4	32	8	8	8	16	8	16	16	16	8
		5	16	8	8	8	16	8	16	16	16	8

xx Antibody titers listed in Table 1.

‡ Fractions containing IgM as detected by radial immunodiffusion.

• C = aliquot of serum-sucrose fraction first treated with buffer

•• 2-ME = aliquot of serum-sucrose fraction first treated with 2-mercaptoethanol.

Table 3. Comparison of whole serum and serum IgM antibody patterns in Americans and Thais hospitalized with presumed Japanese encephalitis.

Patients	Whole serum antibody pattern	Number of serum pairs	IgM HI Antibody Reactivity				Negative **
			JEV ^x			JEV + D ^{xx}	
			R	F ^x	F ^I		
American	1 ⁰ R	7	6	0	0	1	0
	2 ⁰ R	10	9	1	0	0	0
	2 ⁰ F ^x	6	4	1	0	0	1
	Sub-total	23	19	2	0	1	1
Thai	1 ⁰ R	19	16	0	2	0	1
	1 ⁰ F ^x	9	2	1	2	0	4
	2 ⁰ R	27	13	0	1	1	12
	2 ⁰ F ^x	7	0	0	0	1	6
	2 ⁰ F ^I	3	0	0	2	0	1
	Sub-total	65	31	1	7	2	24
Total		88	50	3	7	3	25

x JEV-specific IgM Ab in one or both serum of a pair

xx IgM antibody cross-reactive with JEV and one or more dengue serotypes in one or both serums of a pair.

IgM antibody not detected

Table 4. Cross-reactive IgM antibody patterns in three patients with encephalitis.

Patient Serological Pattern Whole Serum	Day of Disease	Serum Sucrose Fraction+	Reciprocal HI antibody titer+											
			JEV			D4			D3			D2		
			C	2ME		C	2ME		C	2ME		C	2ME	
E 24 yr male American 1 ^{OR}	6	3	2	0		0	0		0	0		0	0	
		4	8	0		2	0		0	0		0	0	
		5	4	0		4	0		0	0		0	0	
F 18 yr male Thai 2 ^{OF} x	16	3	32	0		4	0		0	0		0	0	
		4	>128	0		4	0		0	0		0	0	
		5	>128	0		8	0		2	0		2	0	
G 47 yr female Thai 2 ^{OR}	2	3	16	8		8	4		8	2		8	4	
		4	16	8		8	4		8	4		4	4	
		5	16	8		8	2		8	4		8	4	
G 47 yr female Thai 2 ^{OR}	19	3	16	4		4	0		4	2		2	2	
		4	32	4		8	0		4	2		2	2	
		5	16	4		4	0		4	2		2	2	
G 47 yr female Thai 2 ^{OR}	5	3	2	0		0	0		2	2		2	2	
		4	4	0		0	0		4	2		2	2	
		5	2	0		0	0		4	4		2	2	
G 47 yr female Thai 2 ^{OR}	22	3	32	8		16	8		16	8		16	4	
		4	64	8		16	8		>128	8		64	4	
		5	32	8		16	8		16	8		16	4	

+ See footnotes Table 2

Table 5. Comparison of four serological tests used in the diagnosis of Japanese Encephalitis.

Patient Population	No. Patients with \geq 4-fold JEV monospecific antibody titer rises				
	Whole Serum		Nt	Isolated IgM	HI
	HI	CF			
Thai	19 65	16 61	0 3	31 65	
American	2 23	7 23	3 9	19 23	
Total	19 88	23 84	3 12	50 88	
Total Percent Positive	22%	28%	25%	57%	

xx $\frac{\text{No serum pairs positive}}{\text{No serum pairs tested}}$

Table 6. IgM antibody production in Inapparent JEV and group B arbovirus infections.

Patient Population	Serum HI or CF Antibody titers	Number of Persons Contributing Serum Pairs or Triplicates	Number of Persons with JEV IgM Antibody
Chiangmai valley villagers and urban school children ^x	1 ^{OR} 1 ^{OF} ^x 1 ^{OFI} 2 ^{OR} 2 ^{OF} ^x 2 ^{OFI}	10 1 1 15 2 2	0 0 0 0 0 0
Chiangmai valley villagers ^{xx}	1 ^{OR} 1 ^{OFI} 2 ^{OR} 2 ^{OF} ^x	5 3 1 2	4 0 0 0

^x Bled at 12-16 week intervals.

^{xx} Bled at 2-8 week intervals.

infections characterized by high cross-reactive antibody titers to JEV and to dengue virus serotypes 1-4. Immunoglobulin M (IgM) HI antibody, isolated from whole serum by sucrose density gradient centrifugation, reacted with JEV but not dengue viruses in 31 secondary cases and in 29 primary infection patients. IgM antibody reacted monospecifically with JEV in 60 of 63 patients whose serum contained detectable IgM activity. Moreover, rising JEV IgM antibody titers were found in 6 of 25 patients having fixed or falling whole serum antibody titers. Altogether, 57% of patients were confirmed as recent JEV infections by rising IgM antibody titers, while only 26% were confirmed by conventional serological tests. On the basis of these findings more precise serological criteria have been formulated which utilize whole serum and IgM antibody titrations together for the diagnosis of JEV infections.

2. Japanese B Encephalitis Among United States Marines at Nham Phong Marine Air Base, Thailand, July 1972.

In July 1972 the occurrence of cases of Japanese B encephalitis at a United States Marine Air Base provided an opportunity to study the epidemiology of Japanese encephalitis virus in a population of United States servicemen at risk.

Nham Phong Marine Air Base is located approximately 60 miles south of Udorn, and 30 miles north of Khon Kaen on the Korat Plateau in central Thailand. The area surrounding the base is an elevated scrub forest interrupted by rice paddies approximately 700-900 feet above sea level. The base itself was under construction, the men living in canvas tents. Roads were unpaved, water services consisted of water trailers of potable water brought from a water treatment point. Urine "tubes" and "burn out" latrines were used for human waste disposal. Shower areas were not screened and were operational daily for only limited periods. The men were provided with insect repellent, bed nets, and insect spray.

The base was divided into several discrete living areas (see map Fig 1). The construction battalion (MCB-5) and the logistic support group (LSG) had their own galleys and dispensaries and were located at the north-west end of the runway. Security, MABS-15, and HAMS-15 were located south of the runway and had their meals served on the flight line.

Clinical Cases: Patients seen at the sick bay at Nham Phong with clinical signs and symptoms (fever, headache, stiff neck) suggesting Japanese B infection were examined and blood was drawn for serological testing. Patients were hospitalized at the 432nd Air Force Hospital, Udorn RTAFB. The patients were visited in the

hospital and their clinical charts reviewed.

Seventeen patients from Nham Phong Marine Air Base were hospitalized with a presumptive diagnosis of Japanese B encephalitis. Sixteen patients were hospitalized at the 432nd Air Force Hospital at Udorn RTAF Base. One patient became ill in-transit to Japan and was hospitalized there. Three other patients were seen and followed closely at the Sick Bay at Nham Phong. Fourteen of the total of 20 patients (17 hospitalized, 3 followed as outpatients) demonstrated serological evidence of recent infection with Japanese B encephalitis virus, and of these, eleven had clinical and laboratory evidence of encephalitis.

Serum Survey: A serum survey of randomly selected individuals was begun on 15 July 1972. (From each unit men were selected by randomly choosing final digits of the social security number). Men selected were subsequently identified in either pay lines or unit formations. One month following the original serum collection an attempt was made to re-bleed as many individuals as could be found. The sera were transported to the SMRL for HAI testing against dengue I-IV and chikungunya antigens as well as against JEV.

At the time of the first serum collection in July, 436 men contributed specimens for testing. One month later 351 of these were available for rebleeding and 332 were ultimately re-bled (see Table 1). This represents approximately a 20% sample from the five units that were bled.

Thirty-one persons re-bled had a rise in titer from the first bleed to the second. Fifty-seven persons had a fixed titer in the two specimens of 1:80 or greater. Thus a total of 88 persons of the sample of 332 had evidence of infection with JEV. Thus (assuming the sample is representative) 27% of the men in these five units were infected with JEV during the period of the epidemic. There were approximately 1800 persons in these five units, indicating approximately 480 men were infected. There were 8 cases of clinically recognized encephalitis among the men in these units yielding an apparent to inapparent clinical encephalitis ratio of 1:60. (Weighting the percentage by units would give a ratio of 1:63). (See Table 2).

As can be seen in Table 3 the Security and LSG units had a greater percentage of persons positive for JEV antibody possibly indicating greater exposure. This increased exposure may be explained by night time activity (see Table 3). Security is responsible for patrols around the base at night; almost all of the men in this group may thus have been exposed to the mosquito vectors. LSG, the logistic support group, had men working in bulk fuel and in the ammunition dump at night; thus their exposure

may also have been high.

Rates of non-specific URI illness during the period of the outbreak were similar between those developing JEV antibody and those not developing antibody. Those with no JEV antibody titers had more illness in each category. (See Table 4).

Village Survey: Three Thai villages in the vicinity of the base were visited to assess their possible contribution to the outbreak among the U.S. servicemen. The distances from these village areas to the Air Base are within the flight range of the known vector mosquitoes in Thailand. Each village consists of approximately 70 elevated wooden houses. Each house has animal pens underneath to keep hogs and cattle. The agricultural industry in the area consists of rice, cattle, kenaf fiber, hogs, silk and corn.

There were no known human cases of encephalitis among the villagers at any time before, during, or after the period when cases were appearing on the Air Base. Approximately 60% of the swine in the villages were bled and serologic evidence indicated that transmission in swine had occurred in the area within the previous five months.

Table 1. Units Submitting Serum Specimens

Unit Name	Approximate No. in Unit	No. Bled 1st Bleed	No. Re-bled	% of Unit Re-bled
Security	350	67	54	15%
MABS-15	357	111	76	21%
HAMS-15	588	79	60	10%
LSG	300	54	39	13%
MCB-5	506	125	103	20%
Total	1801	436	332	18%

Entomological Survey: New Jersey light traps were operated inside the theater tent of LSG and in the area of the outdoor theater of the MCB-5 group. Battery-powered CDC traps were run in the

Table 2
JEV Infections by Unit

Unit	No. in Unit	Percent of Men Tested with JEV Infection	Number Estimated JEV Infection	No. Cases	I/A
Security	350	48%	168	1	168/1
MABS-15	357	28%	100	2	50/1
HAMS-15	588	15%	87	1	87/1
LSG	300	43%	130	2	65/1
MCB-5	506	17%	86	3	28/1
Total	1801	26.7%	571	9	63/1

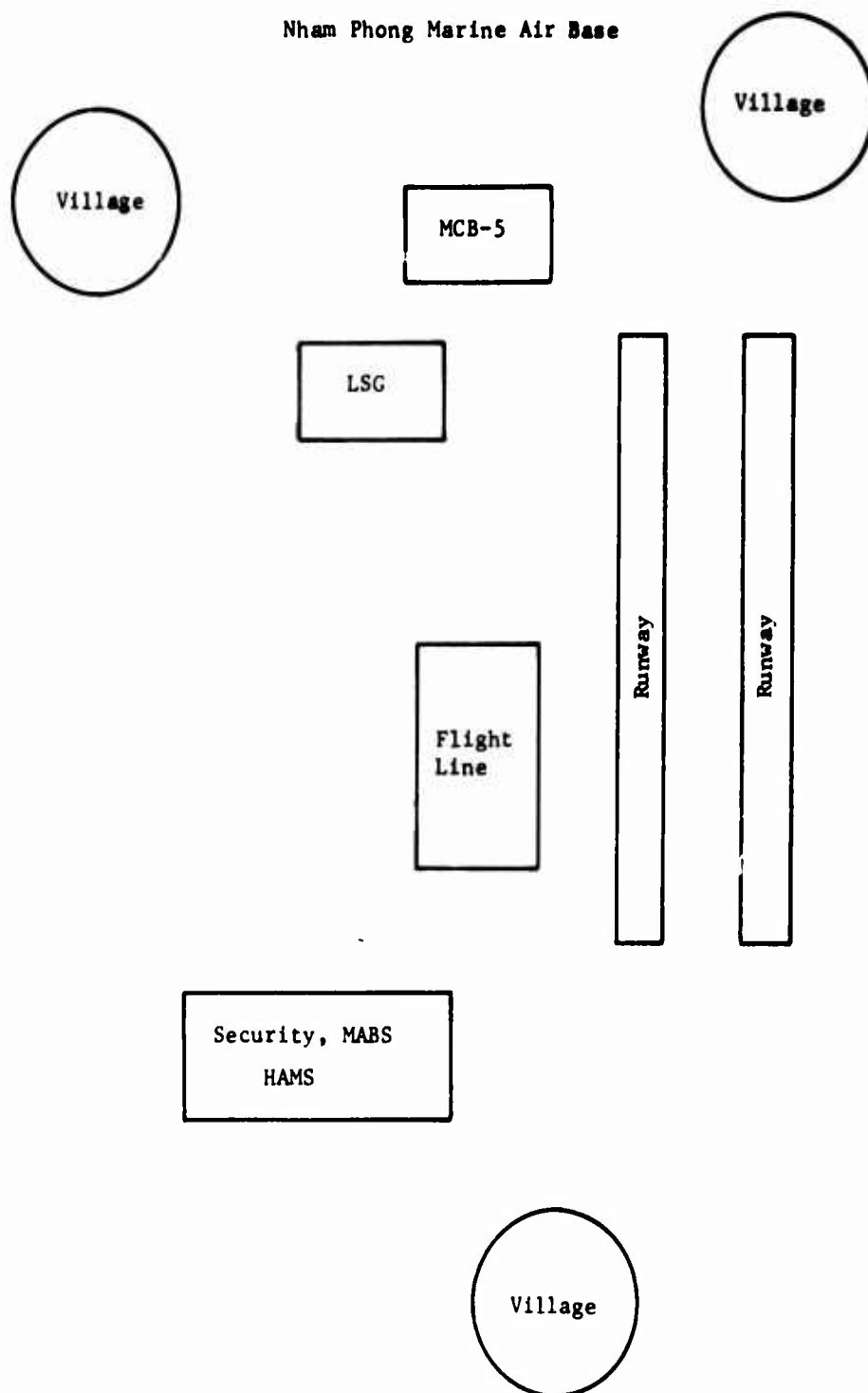
Table 3
Night Exposure by Units

Unit	% with JEV	Average Nites Exposed per Man
Security	48	6.65
MABS-15	28	0.68
HAMS-15	15	0.16
LSG	43	1.26
MCB-5	17	0.98

Table 4
Reports of Illness Among Persons Included in Serologic Survey

Symptoms	With JEV Antibody		Without JEV Antibody	
	#	%	#	%
Total	88	100	246	100
Visits to dispensary in last month	4	4.5	11	4.5
With some complaint	25	28.4	83	33.7
Headache	14	15.9	48	19.5
Cold	10	11.3	37	15.0
Fever	5	5.7	16	6.5
Stiffneck	4	4.5	18	7.3
Headache & Fever	3	3.4	10	4.1

Figure 1
Nham Phong Marine Air Base



vicinity of the LSG showers and the southern end of the runway. CDC traps were also placed in various locations in the villages. These traps were operated for two consecutive nights.

Mosquito collections on the base made on the nights of 19 and 20 July yielded adults of three proven JEV vectors, Culex fuscocephala, C. gelidus and C. tritaeniorhynchus. These and other Culex species were the most abundant mosquitoes in these collections. The drainage system of the base seemed excellent, with little permanent standing water, except for a marshy area receiving waste water which was located about 50 meters west of the LSG showers. Larvae of C. fuscocephala were present in this drainage area.

Mosquito collections in the villages also yielded all three vector species. Larvae of C. fuscocephala and C. tritaeniorhynchus were also collected in the vicinity of each of the villages.

To summarize the above studies, an outbreak of Japanese B encephalitis occurred at Nham Phong Air Base where approximately 3000 presumably non-immune US servicemen were stationed. From the middle to the end of July, 14 clinically apparent encephalitis cases occurred and were sent to Udorn RTAFB Hospital for treatment. It is estimated that approximately 850 inapparent infections also occurred. At this time, in the early rainy season, large numbers of all three vector mosquitoes were present; night duty, night-time outdoor movies, and night-time outdoor showers all contributed to exposing the men to the vector mosquitoes.

None of the villagers in the surrounding Thai villages reported clinical encephalitis. The villages did contain pig herds with 60-80% of the pigs having JEV antibody. In one village, 80% of the pigs under 5 months of age had antibody, indicating transmission in the recent past months. Almost certainly the large number of susceptible pigs, within easy flight range of the vector mosquitoes, performed their now familiar role as an amplifying host.

3. Antibody Levels to Japanese Encephalitis Virus in Swine in an Area of a Human Epidemic

The objective of these studies was to determine and evaluate antibody titers to Japanese Encephalitis Virus (JEV) in swine in the area of a human epidemic.

In July 1972 an outbreak of Japanese encephalitis occurred in American military personnel at the Nham Phong Marine Air Base, Thailand. From previous studies performed by our laboratory in

the Chiangmai area and from studies performed in Japan by other laboratories, swine can be considered an amplifying host for JEV and a potential source of infection for humans through mosquito transmission. Swine can also be used as a sentinel species in following the course of a human epidemic. The villages in the immediate area of the Marine base raise a substantial number of hogs.

Three nearby villages and one pig farm were visited. No clinical cases of encephalitis had been reported in either humans or swine in these locations. One hundred twenty four swine were bled and the fresh whole blood inoculated within 5 minutes into weanling mice in an attempt to isolate the virus of Japanese encephalitis. Sera from the same hogs were tested for hemagglutination inhibition (HI) activity against JEV.

No isolates of JEV were obtained from the specimens taken; however, swine in all four areas showed serologic evidence of previous infection with JEV, with serum antibody titers as high as 1:1280 by the HI method. Table 1 shows the number of animals with positive titers (greater than 1:20) to JEV. In areas 3 and 4, pigs of all ages had a high incidence of positive titers to JEV, but in areas 1 and 2 most animals over 5 months of age showed positive titers while most animals under 5 months of age were negative. Because a few of the young pigs in area 1 exhibited positive antibody titers but most remained susceptible, it was possible that active infection with JEV was present in this area at the time of bleeding. On 30 August 1972, one month after the original sera were collected, areas 1 and 2 were visited again. No clinical cases of JEV had been reported in humans or animals. Sera were drawn from 49 hogs and titers for JEV antibody were determined by the HI method. These results are compared with titers of the earlier sampling in Table 2. Because identification of individual animals was not possible in all cases, sera were not paired but were processed as a group. The incidence of positive JEV titers has not shown any significant change, remaining high (100%) in older animals and low (13%) in the young animals. The epidemic of encephalitis in humans was over; there had been no new cases in the 2 weeks immediately preceeding this second visit.

The epidemic of JEV infection in humans was at its peak before the first serum sampling in hogs was done. The high incidence of positive antibody titers exhibited by very young animals in areas 3 and 4 on 30 July 1972 indicates that these animals were actively infected with JEV only 1 to 2 months earlier and could have served as a reservoir of infection. Passive immunity from the mother might account for some antibody in very young pigs, but does not

Table 1
JEV Titers in Swine from Nham Phong Area

Area	Distance from runway	Approx. Number of Hogs	Hogs Bled	% Positive for JEV Antibody	
				5 Months of Age	5-18 Months of Age
I	1.5 miles North	70	46 (66%)	25% (28)*	89% (18)
II	1.5 miles West	25	15 (60%)	0% (4)	100% (11)
III	2 miles South	60	37 (62%)	81% (27)	100% (10)
IV	5 miles West	190	26 (14%)	42% (26)	

* Total number of animals bled in this group.

Areas:

- I Ban Kong Krung Kli Kuang
- II Ban Kok Sanga
- III Ban Bua Yai
- IV Pig Farm, Nai Koon Punthong

Table 2

Pre and Post Epidemic Titers to JEV in Hogs

Areas	Percent Positive For JEV Antibody		
	< 5 months of age		5-12 months of age
	30Jul72	30Aug72	30Aug72
I	25% (28)*	10% (20)	89% (18)
II	0% (4)	33% (3)	100% (11)
Total	22% (32)	13% (23)	93% (29)
			100% (26)

* Total number of animals bled in this age group.

Areas:

- I Ban Nong Krung Kli Kuang
- II Ban Kok Sanga

account for the high incidence of positive titers among the younger group in areas 2 and 4 as compared to the low incidence of positive titers among the younger group in areas 1 and 2. At the nearby air base were a group of individuals new to Southeast Asia and not previously exposed to JEV. The people at the air base began showing clinical signs of Japanese encephalitis in early July 1972. Villagers who had been in the area a long time had undoubtedly been previously exposed to JEV, probably had a substantial immunity to the infection, and did not develop clinical signs. By the time of our visit on 30 July 1972, the viremic stage in the hogs had already passed, and virus could no longer be isolated from the specimens collected. With this reservoir of virus no longer available, we would expect to see a decrease in clinical cases of JEV in the humans and termination of the epidemic. This is exactly what did happen. From these observations and data, it appears that active infection with JEV occurred first in the hogs in areas 3 and 4, and followed shortly thereafter in the susceptible human population at Nham Phong Air Base. No evidence for active infection with JEV was found in the animals in areas 1 and 2 during the study period. Table 2 indicates that young susceptible hogs in these villages remained susceptible on 30 August 1972, and did not become infected as had the animals in the other villages and the people at the air base. The high incidence of positive JEV titers in animals over 5 months of age confirms the prevalence of JEV in this area. Virtually all individuals had become infected by the fifth month of life. Because hogs are clinically silent when infected, they can exist as an undetected reservoir of JEV.

4. Japanese Encephalitis Virus in Pregnant Swine

The purpose of these studies was to establish whether a relationship exists between Japanese encephalitis virus (JEV) infection and economic problems in swine production in Thailand.

Although JEV usually causes a clinically inapparent infection in swine, evidence from Japan indicates that it is related to problems of infertility, abortion, and the birth of dead or weak piglets. It has become economically advantageous to suspend the breeding of sows in Japan during the encephalitis season so that the losses resulting from this problem are reduced.

The occurrence of JEV among swine and other domestic animals in Asian areas other than Japan is well documented. In two piggeries located near Udorn and Saraburi, Thailand, significant numbers of weak or dead piglets were born from first litter gilts during the encephalitis season of 1971 in herds where serologic examination later showed JEV infection to be endemic. The

productivity of these sows showed great improvement after this first litter. Evidence that JEV is the cause of this problem in Thailand is scanty and only circumstantial; JEV has not been isolated from piglets nor has fetal damage been shown to follow the development of a JEV antibody titer or viremia in the sow. It remains a possibility that the observed problems in swine production are only incidentally related to JEV.

The Kasetsart University pig farm located in Saraburi Province was used as the study site. Previous testing has shown antibody titers to JEV in nearly 100% of the adult animals on this farm. Forty three young gilts were selected for this study, which began in April 1972 and was completed in September 1972. All the animals were bred for the first time during April, May, or June. Sera were drawn from each animal every two weeks and antibody titers to JEV measured by the hemagglutination inhibition (HI) method; titers were run in groups every 2 weeks as collected.

Blood was drawn at weekly intervals and inoculated intracerebrally into weanling ICR mice for attempted virus isolation. The pigs were monitored in this way until they converted (conversion being an eight-fold or greater increase in serum antibody titer) or until after they farrowed. Complete breeding and farrowing records were kept, and the number, health, and appearance of the piglets was recorded.

Only 5 of the 43 animals in the study group still had negative titers at the end of the study; 14 had positive titers when the study began and did not convert, 12 converted before breeding, and 10 converted during gestation. Of the 12 animals showing conversion before breeding, 6 did not conceive after multiple breedings and were sold, and one was bred twice. This can be compared to conception with the first breeding in all 19 animals showing a negative titer and in 12 of 14 animals showing positive titers before the study began. The average litter size in both positive and negative animals was similar, but animals showing JEV titer conversion during gestation suffered from stillbirths and reproductive complications more often than did animals with negative titers. Animals showing negative titers to JEV throughout the study produced an average of 8.60 piglets per litter, those with positive titers produced 7.00, and those converting during the study produced 4.95 healthy piglets per litter. Although a virus was isolated from many of the laboratory animals inoculated, it was usually identified as Ingwavuma virus and is reported elsewhere. JEV was isolated on one occasion on 7 July 1972, the sow aborted at 32 days gestation on 11 July 1972, and conversion was demonstrated on 14 July 1972.

Table 1. Production record of gilts.

Time of JEV Conversion(a)	No. of Animals	Concep- tion Rate	Ave. Litter Size	Healthy Piglets Per Sow	Remarks
2 mo. before breeding	10	40%	9.1	3.90	3 - had normal litters 1 - dystocia-5 piglets suffocated 1 - bred 2 times-3 stillborn 5 - did not conceive in 3 successive breedings
1 mo. before breeding	2	50%	9.0	4.00	1 - stillborn 1 - did not conceive in 3 successive breedings
2nd month of gestation	6	100%	8.8	6.33	4 - had normal litters 1 - 4 stillborn, 2 died the first week 1 - aborted 32 days gestation (e)
3rd month of gestation	4	100%	8.5	6.00	3 - had normal litters 1 - 2 stillborn; sow developed metritis and all piglets died in 1 week
Negative titer (b)	7	100%	9.6	8.60	4 - had normal litters 1 - 5 stillborn 2 - conceived, sow sold for unrelated reasons (d)
Positive titer (c)	14	88%	8.4	7.00	10 - had normal litters 2 - one non-fertile breeding - sold 2 - sow died of unrelated problems (d)

(a) Conversion is an eight fold or greater increase in titer.

(b) Negative titer is 1:20 or less.

(c) Positive titer is 1:80 or greater

(d) These animals were not used in calculating the healthy piglets per sow

(e) JEV was isolated from this sow.

As previously stated, JEV was identified on only one of the isolation attempts. As 22 animals showed JEV titer conversions during the study, it is assumed that these animals were viremic earlier, but that they were no longer viremic on the day samples were taken for attempted virus isolation.

Table 1 indicates that although the average litter size of all groups in the study was similar, sows showing JEV titer conversion during the study had a significantly lower number of healthy piglets than sows not showing conversion. This is true both for animals infected before and during gestation. In the animals which converted before breeding, this is the result of a markedly decreased conception rate. The animals infected during gestation showed a high conception rate, but experienced more complications during gestation and parturition than either animals infected before conception or animals not converting. In both cases, the result was a significant decrease in the number of healthy offspring produced by animals infected with JEV. Animals already showing high antibody titers at the start of the study were similar to those with negative titers throughout the study in that conception rate was high, few reproductive problems were experienced, and a relatively high number of healthy piglets per sow were produced.

Natural infection with the virus of Japanese encephalitis has been shown to reduce the reproductive efficiency of susceptible sows. Serologic and virus isolation studies indicate transmission of JEV in the herd during the study period. Infection immediately prior to breeding appears to reduce the rate of conception, while infection during pregnancy appears to induce a high frequency of complications during gestation and at parturition, particularly a high incidence of stillbirths. The reproductive efficiency was comparatively higher both in sows with high antibody titers not showing evidence of infection near the time of gestation and in sows with negative JEV antibody titers.

5. Ecology of Japanese Encephalitis Virus Infections in Chiangmai Valley: Vector Studies

The purpose of these studies is to investigate the ecology of vectors of Japanese encephalitis virus (JEV) in the Chiangmai Valley, Northern Thailand, with particular emphasis on their dispersal, host preferences and population dynamics.

In studies described in previous Annual Report, JEV was isolated from 3 Culex species - C. fuscocephala, C. gelidus and C. tritaeniorhynchus - in the Chiangmai Valley, and all 3 species

are believed to be acting as JEV vectors in that area. During 1972 studies were undertaken in a village in Saraphi district to determine the pattern of dispersal of vector mosquitoes following the taking of a blood meal. The village selected for these studies was surrounded by rice fields and separated from neighbouring villages by distances of 1-2 Km. On 3 successive nights in July and again in August freshly engorged mosquitoes were collected, counted, marked with a fluorescent dust and released from a central point. Beginning on the second night after release CDC light traps were operated for 7 consecutive nights within the study village and across rice fields in neighbouring villages. All mosquitoes collected in these traps were examined under a longwave ultraviolet lamp for marked specimens.

To determine if the density of vector mosquitoes varies according to the distance from blood meal sources, CDC light traps were operated at intervals across an open rice field from a village in Mae Rim district. Five collection sites were selected; the first trap was located within the village near livestock and the other four at increasingly greater distances from the village across 1700 meter wide rice fields. The last collection site was more than half the distance across the rice fields to another village. Studies were also made to determine if the JEV vector species demonstrate seasonal differences in host preferences. Three Magoon traps were baited on successive nights each week with a buffalo, cow or pig. These animals were rotated from trap to trap to eliminate differences due to trap location.

In July 1972 an estimated 12,723 C. fuscocephala, 444 C. gelidus and 21,380 C. tritaeniorhynchus were marked and released in Saraphi district. In CDC trap collections made following their release 27 C. fuscocephala (0.21%) and 40 C. tritaeniorhynchus (0.18%) were recaptured. Both species apparently dispersed in a random fashion and marked specimens were recaptured at distances up to 1400 meters from the release point (Figure 1). For unknown reasons the majority of marked mosquitoes were recaptured at one site 1100 meters west of the release point. In August an estimated 6,078 C. fuscocephala, 481 C. gelidus and 10,718 C. tritaeniorhynchus were marked and released from the same village site. Light traps were operated for the same period and at the same sites as in the first experiment. A total of 15 C. fuscocephala (0.24%), 1 C. gelidus (0.20%) and 2 C. tritaeniorhynchus (0.02%) were recaptured in this experiment. The dispersal pattern was similar to that observed in the first experiment (Figure 2). However, 1 C. tritaeniorhynchus was recaptured at a point 1800 meters from the release point, which was the maximum distance at which light traps were located from the release point. No

Table 1 Summary of mosquito collections in CDC traps set in a rice field at various distances between two villages - Chiangmai, Thailand, 1972-73.

		TN=147		TN=70		TN=70		TN=70		TN=70	
Location		In First Village near animals		175 m From First Village		477 m From First Village		741 m From First Village		663 m From Second Village*	
Species	Sex	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀
<u>C. fuscocephala</u>		698	55550	8	115	52	36	0	3	0	14
<u>C. gelidus</u>		32	3100	3	19	1	3	0	2	0	0
<u>C. tritaeniorhynchus</u>		493	86897	4	245	18	68	0	9	1	26
Vector Species/ Trap Night		8.3	990.1	0.2	5.2	1.0	1.5	0.0	0.2	0.01	0.6

* = 1037 meters from 1st village.

TN=Number of Trap Nights

correlation between the pattern of dispersal of vector mosquitoes with either wind direction or velocity was observed during these experiments. The flight ranges of these mosquitoes would allow for rapid dissemination of JEV from village to village in the Chiangmai Valley.

The results of CDC trap collections made at varying distances across rice fields from a village in Mae Rim district are summarized in the Table 1. Statistically significant differences were observed between numbers of mosquitoes collected in the village and in the rice fields. These differences appear to be directly correlated with the distance of trap locations from the nearest village.

From September 1972 to February 1973 larger numbers of Culex fuscocephala, C. gelidus and C. tritaeniorhynchus were collected from Magoon traps baited with a buffalo than from the traps baited with either a cow or pig. Lowest numbers were collected in December and January with populations beginning to increase in February. The numbers of mosquitoes in cow-baited traps fluctuated in the same general pattern as those in the buffalo-baited traps. Consistently fewer mosquitoes were collected in pig-baited traps than when either a buffalo or cow was used, however, some specimens of each of the 3 species were collected each month in the traps baited with the pig.

C. ARBOVIRUSES

1. Isolation of Ingwavuma Virus from Domestic Pigs

Detailed in the 1971-1972 SMRL Annual Report was the isolation of Ingwavuma from a Culex vishnui pool obtained in the Chiangmai Valley in 1970 and serologic evidence that domestic pigs indigenous to the valley were commonly infected with Ingwavuma (or a closely related virus). Studies undertaken during the past year have established Ingwavuma antibody prevalence in areas of Thailand other than the Chiangmai Valley, have demonstrated natural infection of Thai pigs with Ingwavuma virus, and have extended the range of this virus eastward to Taiwan.

Prevalence of Ingwavuma Antibody in Thai Pigs from Areas Other Than Chiangmai: Sera of 20 pigs from each of 3 areas of Thailand-Kanchanaburi in the West, Udorn in the Northeast, and Saraburi in the Central plains-were tested for neutralizing (NT) antibody at a 1:20 serum dilution by the micro-plaque reduction neutralization test (PRNT) described in last year's annual report. Serum was considered to have antibody if it reduced the number of plaques to

less than 80% of the control plaque counts. Ingwavuma antibody was found in pigs from all 3 areas, suggesting a broad distribution of this virus in Thailand; the prevalence was 50% in pigs at Kanchanaburi, 45% at Saraburi, and 10% at Udorn.

Natural Infection of Thai Pigs: In March 1972, the Department of Veterinary Medicine began a study at the Tubkwang Breeding Farm of Kasertsart University in Saraburi Province to determine the effects of Japanese encephalitis infections in pregnant sows. Beginning in April, serum for viral serology was obtained bi-weekly on study animals and weekly blood specimens were inoculated directly into weanling mice which were transported to SMRL and observed. Brain suspensions of dead or sick mice were further passaged in suckling mice.

Twenty-eight pregnant sows were thus studied from April through July 1972. No Ingwavuma isolates were obtained from the 6 animals who had NT antibody at the start of the study. Of the 22 animals initially lacking NT antibody, 16 had serologic evidence of infection and isolates were recovered from 11 (Table 1). Only one isolate was obtained per animal, suggesting that the duration of Ingwavuma viremia in pigs is less than one week. All but 2 of the isolates were from blood collected in the 2 week period from 21 April through 7 May. Significant rainfall occurred in Saraburi in March and April. Convalescent antibody titers of infected pigs ranged from 1:160 to $\geq 1:640$, the geometric mean titer was 1:360.

Ingwavuma Isolates from Nam Phong, Thailand, and Taiwan: In addition to the Saraburi isolates, 3 strains of Ingwavuma virus were isolated from blood obtained from pigs near Nam Phong Air Force Base in the Korat Plateau. Three virus strains isolated from pig blood in Taiwan by Dr. Ananda Nisalak of NAMRU 2 have been shown to be identical to Ingwavuma virus by PRNT with hyperimmune BKM 705 antisera.

Isolation studies undertaken during the past year have confirmed the hypothesis developed from past antibody prevalence studies that pigs are an important host of Ingwavuma virus in Southeast Asia. Our studies have expanded the range of this virus previously isolated only in Africa and India through Thailand to Taiwan. Further studies of the virus which rarely if ever infects humans are not planned.

Table 1. Natural Ingwavuma Infections in Pigs, Saraburi, 1972

Ingwavuma NT Antibody Status		Number of Pigs	
March 1972	July 1972	Studied	With Ingwavuma Isolates
Absent	Present	16	11
Absent	Absent	6	0
Present	Present	6	0

2. Survey of Tick-Borne Viruses in Thailand

Over 50 species of ticks have been collected by SMRL personnel in the past few years from 37 provinces of Thailand; 10 of these species have been recorded as attacking human beings. Several arbovirus diseases of public health importance are transmitted to man through ticks. Most prominent have been the tick-borne encephalitis complex of Group B arboviruses, Congo group arboviruses and the Kemerovo group viruses. Viruses of these groups have been defined as etiologic agents in outbreaks of meningo-encephalitis and hemorrhagic fever. No surveys for tick-borne viruses have previously been made in Thailand, and only two arboviruses - Nyamanini and Pathumthani viruses - have been isolated from ticks collected in Thailand.

During this period a total of 10,888 ticks were collected from six provinces (i.e., Chiangmai, Mae Hong Son, Nakhon Nayok, Nakhon Ratchasima, Pathumthani and Prachinburi), identified and pooled for virus isolation attempts. Included in the 709 pools tested were 28 species of ticks - belonging to the genera Amblyomma, Aponoma, Argas, Boophilus, Dermacentor, Ixodes, Haemaphysalis and Rhipicephalus (Table 1). The largest numbers of ticks tested belonged to the genera Boophilus and Haemaphysalis, respectively. Various collection techniques were employed during this period, but the largest numbers of ticks collected were removed from trapped wild mammals and birds and from domestic animals. A total of 1205 small mammals and 262 birds were trapped and examined for ticks. Approximately 400 domestic animals, including buffalo, cattle, goats and dogs, were examined for ticks. Finally, 2262 ticks were collected resting on vegetation or in the vicinity of avian rookeries.

Six unidentified, ether sensitive viral agents were isolated from pools of Argas robertsi collected in the vicinity of nests of Night Herons (Nycticorax nycticorax) in Nakhon Nayok province. Thus far, no viruses have been recovered from ticks removed from mammals or birds trapped during this period.

Table 1. Ticks collected in Thailand and tested for viral agents, 1972-73.

Species	No. of Pools	No. of Ticks
<u>Amblyomma</u> sp.	7	16
<u>A. testudinarium</u>	1	1
<u>Aponomma</u> sp.	3	11
<u>A. lucasi</u>	2	28
<u>Argas</u> sp.	0	0
<u>A. pusillus</u>	3	90
<u>A. robertsi</u>	12	130
<u>Boophilus</u> sp.	2	5
<u>B. microplus</u>	197	2,931
<u>Dermacentor</u> sp.	1	4
<u>D. auratus</u>	22	49
<u>D. atrosignatus</u>	4	6
<u>Ixodes</u> sp.	5	10
<u>I. granulatus</u>	20	33
<u>Haemaphysalis</u> sp.	52	2,661
<u>H. anomala</u>	20	567
<u>H. atherurus</u>	1	2
<u>H. bandicota</u>	104	1,177
<u>H. canestrinii</u>	1	4
<u>H. cornigera</u>	18	563
<u>H. heinrichi</u>	75	1,106
<u>H. lagrangei</u>	12	145
<u>H. obesa</u>	6	12
<u>H. semensis</u>	6	130
<u>H. wellingtoni</u>	7	19
<u>Rhipicephalus</u> sp.	38	176
<u>R. h. haemaphysaloides</u>	71	693
<u>R. sanguineus</u>	19	319
Total	709	10,888

D. INFLUENZA

1. An Outbreak of Influenza A among USAF Personnel at Udorn Royal Thai Air Force Base.

On 19 September 1972 this laboratory was requested by the 1st Medical Service Wing (PACAF) to assist in the investigation of an outbreak of febrile upper respiratory tract disease at Udorn Royal Thai Air Force Base, Thailand. An increase in URI dispensary visits was first noted on 10 September and by 17 September 53 flight crew members were grounded with a flu-like syndrome. The explosive nature of this outbreak, its impairment of the mission of the base, and its occurrence in a military population previously immunized with polyvalent influenza vaccines necessitated its immediate investigation. Accordingly, SMRL investigators visited Udorn RTAFB on 20 September. It was decided that epidemiologic investigation and institution of control measures were the responsibility of the 1st Medical Service Wing while etiologic investigation of the outbreak was the responsibility of SMRL.

Etiology: Preliminary investigation established that approximately 300 men had developed the syndrome in the two days preceding 20 September, and that most units on the base were involved. Eighteen USAF personnel hospitalized or visiting the emergency room of the 432nd USAF Hospital were examined by SMRL investigators. Clinically these patients presented almost universally with a dry, hacking cough, sore throat of variable degree with marked pharyngeal injection but no exudate, marked malaise, headache, and myalgias. Systemic symptoms predominated. Oral temperatures ranged from normal to 105°F; all but 3 of the 18 had temperatures greater than 100°F. White counts on these patients were generally between 8,000 and 12,000.

Throat swabs obtained on the 18 patients were immediately plated and streaked on sheep blood agar plates. Small numbers of B hemolytic streptococci were isolated from but two patients and one of these isolates was Bacitracin resistant.

Throat washings in Hanks' balanced salt solution and 0.4 percent bovine plasma albumin were obtained on all 18 patients, immediately frozen on dry ice, transported to SMRL and inoculated into embryonated eggs and rhesus monkey kidney cell cultures. Strains of influenza A were isolated from throat washings of 13 patients - from all 6 hospitalized patients and from 7 of the 12 patients examined in the emergency room.

Acute serum was obtained on all 18 patients and 3 week convalescent serum was obtained by the 432nd USAF Hospital on 10 of these patients. Of those 7 patients with influenza A isolated, 6 had

four-fold or greater rises in hemagglutination-inhibition (HI) antibody to one of the Udorn isolates, A/Udorn 302/72. None of the three patients without isolates had diagnostic antibody rises.

Antigens of one of the Udorn isolates, Udorn 302, were compared with previous influenza A strains in cross HI tests. Rooster antisera to A/JAP 305/57, A/HK 1/68, and A/Udorn 302/72 were used in these tests against A/JAP 305/57, A/HK 1/68, A/Korat/72 (a strain isolated in Korat, Thailand, in February 1972), and A/Udorn 302/72. Results are shown in Table 1. The data indicate that A/Udorn 302/72 is related to strains represented by A/HK 1/68, but is different antigenically from the 1968 and 1972 strains. Rooster antisera to these latter strains were but weakly reactive to the Udorn strain. This would suggest that human antibodies induced by previous infection or immunization with 1968 antigens would afford considerably less protection to the Udorn strain than to the 1968 or Korat 1972 strain.

Throat washings and strains isolated from the Udorn patients were forwarded to the WRAIR for more detailed antigenic analysis and for possible vaccine use. Although final reports have not been received on the Udorn isolates, a strain isolated in Bangkok in September 1972, A/Bangkok 2/72 (which appears antigenically identical to A/Udorn 302/72), has been characterized as identical to A/England 42/72 by the WHO Influenza Reference Laboratory in England.

Epidemiology: Between 10 September and 30 September, 1387 USAF personnel reported to base medical facilities with URI or influenza-like symptoms. All patients were examined by physicians or corpsmen; those seen after 20 September filled out a questionnaire at the time of examination while those examined before 20 September were contacted and questionnaires filled out retrospectively. The questionnaires specifically dealt with squadron, clinical symptomatology, and date of last influenza immunizations. Pertinent findings from this data obtained by 1st Medical Service Wing are summarized.

Total numbers of suspected influenza cases seen at Udorn RTAFB in September 1972 are shown in Table 2. The attack rate for total base personnel was 21%, but considerable variation between units was observed. Of interest was that 62% of the base flying personnel developed illness as opposed to 16% of non-flying personnel.

Dates of onset of involved personnel are shown in Figure 1. The explosive nature of the 1972 Udorn outbreak was in contrast with that observed by Smith, et al, in an A/HK/68 outbreak at Korat RTAFB in 1968 (SMRL Annual Report, 1969).

The frequency of individual symptoms in the 1387 patients studied are shown in Table 3. Sore throat, coryza, fever, headache, and

malaise were found in over 2/3 of the patients, while cough and myalgias were complaints of about 50%.

A history of previous immunization with polyvalent influenza vaccines was obtained in 91% of the 1387 patients studied; 88% of all patients had received immunizations on or after October 1971. Although the data obtained do not permit a comparison of attack rates in immunized versus unimmunized personnel, the available data suggests that influenza vaccines containing A/HK/68 antigens were at best of limited effectiveness against the 1972 Udorn strain.

Table 1. Comparative HI Tests with A/Udorn 302/72 Antigen and Early Influenza Isolates

Antigen	A/JAP 305/57	A/HK 1/68	A/Udorn 302/72
A/JAP 305/57	<u>40</u>	< 20	< 20
A/HK 1/68	40	<u>320</u>	80
A/Korat/72	20	<u>160</u>	80
A/Udorn 302/72	20	40	<u>160</u>

Table 2. The Totals of Influenza Cases at Udorn RTAFB during September 1972 Listed by Unit of Assignment

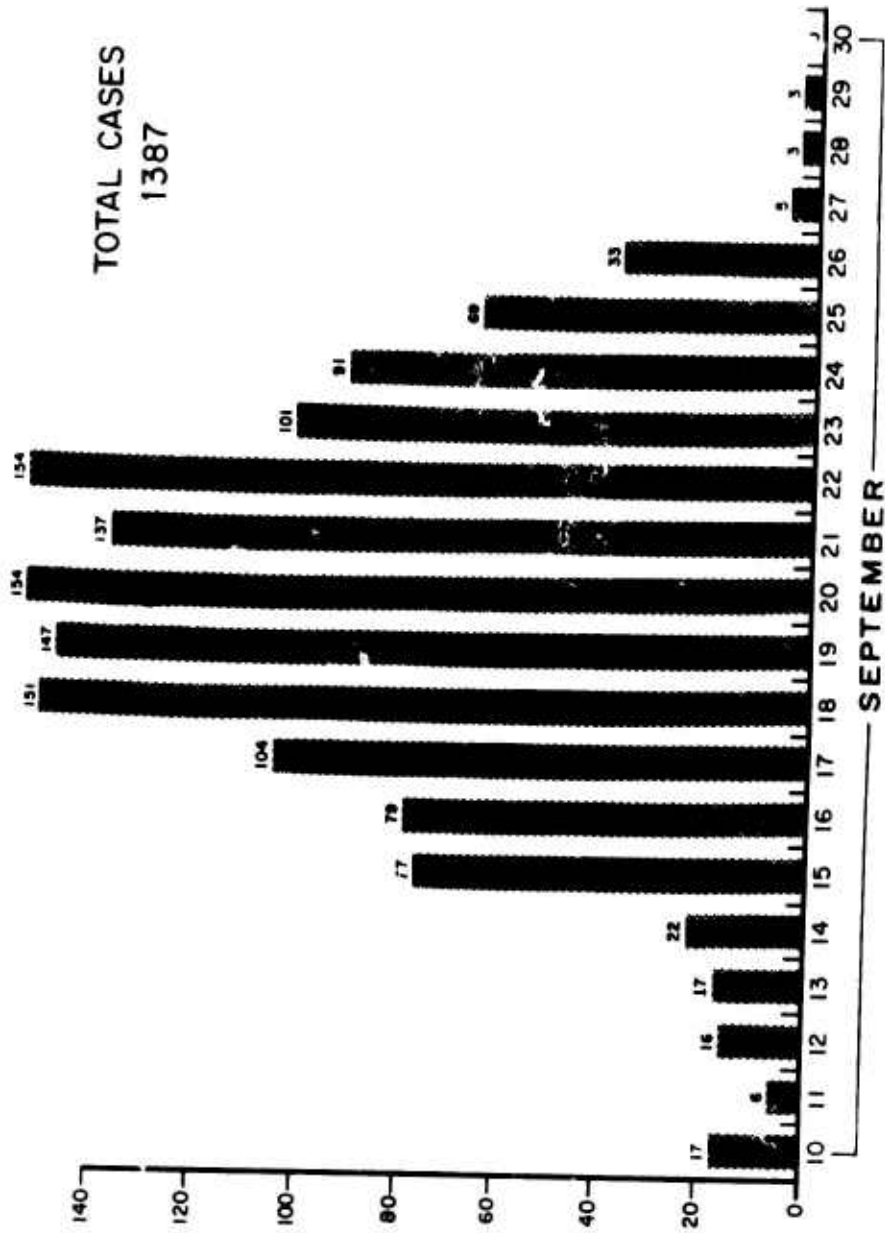
Unit	Population*	Cases	Attack Rate (%)
58 TFS	100	68	68
432 SPS	250	146	58
555 TFS	75	39	52
13 TFS	75	31	41
14 TFS	125	50	40
Det 1, 7AF (7/13 AF)	50	16	32
432 FMS	450	141	31
432 AMS	350	97	29
432 MMS	300	79	26
432 CES	150	35	23
432 OMS	300	70	23
432 USAF Hospital	125	27	22
621 TCS	125	24	19
1974th Comm Sq	350	62	18
432 CSG	2000	341	17
432 Supply Sq	250	41	16
Det 5	15	2	13
307th TFS	450	54	12
56 SW Det 1	300	31	10
523rd TFS	400	26	7
Misc Small Units	400	7	3
TOTALS	6640	1387	

* All unit strengths are rounded to nearest twenty-five.

Table 3. A Comparison of Symptom Proportions in Influenza Cases
Interviewed at Udorn RTAFB During September 1972

Symptoms	Yes	No	Percentage with Symptom
Sore Throat	1140	247	88
Runny Nose	1080	307	87
Fever	1095	292	79
Less than 102°	1095	292	79
More than 102°	319	1068	23
Headache	1054	333	76
Malaise	943	444	68
Chills	874	513	63
Cough	721	666	52
Myalgia	600	787	47
G.I. Symptoms	443	944	32
Arthralgias	374	1013	27
Red Eyes	374	1013	27
Rash	34	1353	5

Figure 1. THE DATE OF PATIENTS' FIRST SYMPTOM DURING INFLUENZA OUTBREAK AT UDOR, SCAPF I, SEPTEMBER 1972



E. RABIES

1. Rabies Diagnostic Laboratory Service in Thailand.

This laboratory provides accurate, rapid rabies diagnostic service for U.S. military units in Southeast Asia and in the Western Pacific. The laboratory utilizes the fluorescent antibody test and mouse inoculation on all cases received. All reports are made via telephone and are followed by written confirmation.

During the period 1 April 72 to 30 March 73 brain specimens of 1016 animals were received for rabies examination (Table 1). The dog continues to be the most frequently infected species in Thailand. The percentage of positive cases of canine rabies is essentially the same as it has been in previous years of this laboratory operation.

Table 1. Summary of Rabies Diagnoses 1 April 1972 - 31 March 1973

Species Examined	Number Examined	Number Positive	Percent Positive
Canine	787	394	50.1
Feline	132	24	18.2
Rodent	41	0	0.0
Nonhuman Primate	18	0	0.0
Bat	11	1	9.9
Rabbit	9	0	0.0
Human	6	3	50.0
Equine	5	0	0.0
Mongoose	2	0	0.0
Porcine	1	1	100.0
Bovine	1	1	100.0
Ovine	1	0	0.0
Civet	1	0	0.0
Otter	1	0	0.0
Total	1016	424	41.7

2. The Use of the Corneal Test as an Ante-mortem Method of Rabies Diagnosis in Human Patients.

There have been reports of the successful application of the fluorescent antibody technique to corneal impression smears from experimentally infected animals (Schneider, L. G., 1969, Zentralbl. Vet. Med , 16, 24-31). There is at least one report of the successful

use of this procedure in the ante-mortem diagnosis of a human case of rabies. (Cifuentes, E., et al, 1971, J. Trop. Med. Hyg. 74, 23-25).

Recently, we have performed the corneal test on two human rabies patients. The first patient was a 43 year-old woman who had been bitten by a rabid dog 3 weeks prior to the onset of symptoms. Corneal impressions were taken before and after death, and in both instances positive immunofluorescence was detected. Postmortem examination of the brain revealed positive immunofluorescence as well as the presence of Negri bodies in formalin-preserved samples. The second case involved a 22 year-old American soldier who had been bitten by a rabid dog 5 weeks before the onset of symptoms. Positive immunofluorescence was detected in corneal impressions taken 11 days before death. Post-mortem corneal samples were also positive. The diagnosis of rabies was confirmed by the observation of positive immunofluorescence and Negri bodies in the brain after death.

Considering these encouraging initial findings, critical evaluation of the corneal test in human rabies patients is warranted. A research proposal for this purpose has been developed. At least twenty human rabies patients will be studied to determine the reliability, specificity, and time of development of corneal immunofluorescence.

3. Reservoirs of Rabies in Thailand.

a. Survey of Wild Rodents: A survey was performed to determine the prevalence of rabies virus infection in wild rodents in the Choburi-Rayong area. This area was selected because: 1) there was a high incidence of canine rabies, and 2) a high frequency of rabies virus infection was reported in bandicoots (Bandicota indica) in this area in 1966 (Smith, P. C. et al, Nature 217:954, 1968).

A total of 704 wild rodents, 520 of which were bandicoots, were captured in the Choburi-Rayong area during the period Sep 72 through Dec 72. The brains were removed, frozen, and stored for later examination for rabies virus using the fluorescent antibody technique.

To date, 200 rodent brains have been examined using the fluorescent antibody technique and mouse inoculation, and all were found to be negative for rabies. The remaining rodent brains will be examined for rabies virus infections during calendar year 1973.

b. Ancillary Studies in Wild Rodents: Wild rodents captured in the rabies survey described above were examined at necropsy, and each animal was examined for enteric and blood parasites. The results of the parasitologic examinations are reported elsewhere in this volume. Bacteriologic examinations of lung, liver, kidney, nasopharyngeal swabs, and feces were performed on 167 animals. In one Rattus rattus, a non-agglutinating Vibrio sp. was isolated. No other significant

bacterial pathogens were isolated. Visceral organs from all animals were preserved in 10% neutral buffered formalin, and histopathologic studies are in progress.

c. Survey of Dogs for Rabies Virus Infection: A total of 385 canine brain specimens were obtained from the Bangkok Municipal Health Department dog pound and from cooperating U.S. Air Force installations in Thailand. The U.S. Air Force specimens were obtained in collections of stray dogs on the bases. None of these dogs were yet manifesting clinical signs suggestive of rabies at the time of sacrifice. Each specimen was examined by the fluorescent antibody method. Positive cases were confirmed by mouse inoculation.

The results of the fluorescent antibody examinations are shown in Table 1. The overall infection rate of 3.4% is within the 2-6% range reported in previous surveys during the past 5 years.

The results of these surveys indicate that rabies continues to be a significant public health problem among the stray dogs in Thailand.

Table 1. Isolation of Rabies Virus from Stray Dogs in Thailand.

Source	No. Specimens Examined	No. of Rabies Virus Isolations	Percent
Korat	89	4	4.7
Udorn	72	2	2.8
Ubon	50	0	0.0
Nakorn Sawan	38	1	2.6
Nakorn Panom	36	2	5.6
Bangkok	<u>100</u>	<u>4</u>	<u>4.0</u>
Combined	385	13	3.4

F. HEPATITIS B

1. A Comparison of Complement Fixation, Immuno-electrophoresis and Solid Phase Radioimmunoassay for the Detection of Hepatitis B Antigen in Thai and American Populations.

The objective of these studies was to develop the radioimmunoassay (RIA) for detection of Hepatitis B antigen (HB-Ag) and to compare the sensitivity of this assay with that of other tests for antigen used previously in this laboratory.

In previous years HB-Ag has been detected in sera submitted to this laboratory by three methods: agar gel diffusion (AGD), immuno-electroosmophoresis (IEOP) and complement fixation (CF). Over the past several years the prevalence of Hepatitis B antigen has been determined by these methods in several different Thai and American populations. These populations include groups of United States military personnel entering the Republic of Vietnam, U.S. military personnel suffering from acute hepatitis in the RVN, Thais living in a low income urban subdivision of Bangkok, paid Thai blood donors at Phra Mongkut Klao Hospital and Thai children with acute hepatitis seen at the outpatient clinic at Bangkok Children's Hospital. Because of the increased sensitivity attributed to the solid phase radio-immunoassay, the sera collected from these populations were submitted for HB-Ag testing by this method.

The sera tested were taken from the six groups described above. The method for AGD, CF and IEOP have been presented before (1971-1972 Annual Report, SEATO Medical Research Laboratory).

Reagents for the solid phase radioimmunoassay were obtained from Abbott Laboratories, Chicago, Ill. as Ausria kits. The tests were performed according to the instructions provided. Briefly 1 ml of a sample to be tested was placed on the bottom of a plastic tube coated with guinea pig HB-Ag antibody. The tube was stoppered and allowed to incubate at room temperature for 16-18 hours (overnight). The sample was then removed and the tube rinsed 5 times with 2 ml each of 0.01 M Tris-HCl, pH 7.1. One tenth milliliter of ¹²⁵I-labeled HB-Ag antibody solution (0.1 uCi) was then delivered to the bottom of the tube. After the tube was capped and allowed to incubate at room temperature for 90 minutes, it was aspirated and rinsed five times as above. Seven negative control tubes were prepared in the same manner with a standard normal human plasma provided by Abbott Laboratories. Each of the tubes was counted in a gamma ray spectrometer and the mean and standard deviation of the serum negative control tubes were computed. The coefficient of variance of these negative control tubes was between 10 and 20%. Samples were considered positive if they were greater than five standard deviations above the mean of the negative control value. Samples equal to or less than five standard deviations above the mean were considered negative.

Tables 1 and 2 illustrate the increased sensitivity of the RIA over the CF and IEOP. Of a total of 1638 samples tested 145 or 8.8% were positive by CF, 197 or 12.0% were positive by IEOP and 233 or 14.2% were positive by RIA. The increased sensitivity of the RIA over the CF in the total population was 88/233 or 38% while the increased sensitivity of the RIA over the IEOP was 40/233 or 17%.

That the RIA is capable of detecting HB-Ag later in the course of antigen positive hepatitis than other methods was demonstrated in

a longitudinal study of five Thai children with HB-antigen positive disease. In one child followed for one year, the CF, IEOP & RIA remained positive, and this child was considered a carrier. In two cases followed for periods of time up to 8 months, the RIA remained positive beyond the IEOP or the CF for 1-3 months. Follow-up sera collected on both American and adult Thai hepatitis patients are at present under investigation.

A breakdown of the total population tested by RIA into component groups is shown in Table 3. It will be noticed that the RIA increases the detection of antigen in all of the groups tested; however, when used to detect HB-Ag on samples obtained from American personnel the RIA detected 3 to 4 times as many additional positive samples as when it was used to study Thai populations. Further analysis of histograms, formed by plotting the counts/minute or standard deviations above control mean on the abscissa and number of persons studied on the ordinate, showed different curves for American and Thai HB-Ag positive samples. The Thai populations showed a unimodal curve with its mode greater than 100 standard deviations from the mean. The American military hepatitis patients showed a bimodal curve with peaks at about 50 and 100 standard deviations from the standard control mean.

The increased sensitivity of the radioimmunoassay over previous methods of detecting hepatitis B antigen is amply demonstrated by these data. The RIA not only detects more hepatitis B positive samples in point prevalence studies but also allows for the detection of HB antigen for longer periods in the convalescence from HB antigen positive disease.

A number of explanations may be offered for the differences in concordance of RIA with IEOP between Thai and American subjects. It might be that the RIA in our hands is less sensitive than it is elsewhere. This seems unlikely since results of the RIA done in this laboratory compare favorably with determinations performed on aliquots of the same Thai samples by the laboratory of Dr. Roger Dodd, Red Cross Research Laboratory, Bethesda, Maryland.

It might be that Thais carry antigen at a higher titer than do carriers in temperate zones. The majority (45 of 62 or 73%) of the Thai subjects in the lower socioeconomic subsection who manifested antigenemia by the radioimmunoassay had complement fixation titers of 1:64 or greater as did all of the HB-Ag positive blood donors tested. In the American soldier population only 1 of 5 (20%) had an antigenemia of this titer; however, despite the high CF titer in carriers, there were also relatively small differences between the number of IEOP and RIA positive Thais with hepatitis despite a large number (81%) of low or negative titer CF tests.

A third and most likely explanation is that the higher concordance

Table 1. CF & RIA Results for Total Population

RIA Test	CF Test		TOTAL
	NEG	POS	
NEG	1405	0	1405
POS	88	145	233
TOTAL	1493	145	1638

Table 2. IEOP & RIA Results for Total Population

RIA Test	IEOP Test		TOTAL
	NEG	POS	
NEG	1405	0	1405
POS	40	193	233
TOTAL	1445	193	1638

Table 3. A Comparison of CF, IEOP, & RIA Results in the Detection of HB-Ag, in Several Thai and American Populations

	Total	CF	IEOP	RIA	Increased detection** of RIA over IEOP
U.S. Military Entering S.E.A.	500	2(0.4)*	3(0.4)	5(1.0)	40 %
U.S. Military Hepatitis	174	62(36)	68(39)	94(54)	28 %
Thai Lower Class Population	687	47(7)	55(8)	62(9)	10 %
Thai Blood Donors	100	8(8)	9(9)	9(9)	0 %
Thai Adult Hepatitis	113	21(20)	51(45)	54(48)	10 %
Thai Children Hepatitis	64	5(8)	8(13)	9(14)	4 %

* Number positive (/ of total positive)

** Number of IEOP false negative/total RIA positive x 100

between IEOP and RIA tests in Thais as opposed to Western populations is related to the homogeneity of HB-Ag in Thais and the heterogeneity in U.S. personnel. All antigens thus far identified in this laboratory from the Thai population have been subtyped ad and the majority of them have been adr (see elsewhere in this report). Subtyping of the sera from American hepatitis B carriers and hepatitis patients showed that 50% of samples (18 of 36) were ayw.

Both the CF and the IEOP are performed in the laboratory using Thai human HB-Ag antibody which has been shown to have anti-ad activity but no anti-y activity. Because of the specificity of antibody for the Thai antigen tested, a high sensitivity of the IEOP and the CF test is possible. On the other hand, the HB-Ag subtypes of American populations are heterogeneous, therefore the specificity of the Thai ad antibody is less and the sensitivity of the test is reduced.

The RIA utilizes a guinea pig antibody against HB-Ag. This antibody pool has been found to have a much greater anti-ad activity than anti-y activity and for this reason should produce higher counts/minute with equal amounts of ad versus ay antigen. The bimodal curve that was seen when the counts of sera from American hepatitis patients were plotted may indicate that the test is reading two subtype populations while the plot of the data from Thai subjects suggest that only a single antigen population ad is being detected.

2. The Epidemiology of Hepatitis B Antigen: A Defined Urban Thai Population; Antibody Acquisition.

For a description of the experimental design of this study see the SEATO Medical Research Laboratory Annual Progress Report, 1971-1972, pp. 129-139. Data accrued as to the point prevalence of antigen in this population indicate that infection is acquired early in childhood. The early acquisition of the carrier state and the high prevalence of the antigen suggest a high rate of HB-Ag transmission through this population. Studies of antibody prevalence were undertaken in a random sample of urban Bangkok residents to determine the prevalence of antibody to hepatitis B antigen (HB-Ag).

Sera collected from this population were submitted to the laboratory of Dr. Roger Dodd, Red Cross Research Laboratory, Bethesda, Maryland, U.S.A., for antibody assays by the passive hemagglutination method described by Vyas and Shulman.

Preliminary reduction of the data on the prevalence of HB-Ag antibody among this population indicates that 311 of 655 (47%) had antibody detectable by the passive hemagglutination test (see Table 1). Breakdown of this data into age specific antibody prevalences demonstrates a 14% prevalence of antibody in the 1-4 year age group and a rapid acquisition of antibody to a plateau of about 60% in the

Table 1

Prevalence of Hepatitis B Antibody by Age in Urban Bangkok Residents

Age	MALE			FEMALE			TOTAL		
	No. Tested	HBAb(+)		No. Tested	HBAb(+)		No. Tested	HBAb(+)	
		No.	%		No.	%		No.	%
1-4	32	6	19	19	1	5	51	7	14
5-9	49	15	30	51	13	25	100	28	28
10-14	58	25	43	63	23	37	121	48	40
15-19	38	23	67	52	20	38	90	53	59
20-29	38	24	63	67	29	43	105	53	50
30-39	26	20	78	51	31	61	77	51	66
40	42	27	64	69	44	64	111	71	64
Total	283	140	49	372	161	43	655	311	47

ages above 20.

This data is undergoing statistical analysis at the time of this writing with a view towards early publication.

3. Observations of HAA in a Blood Donor/Recipient System in Thailand.

This is a continuation and progress report on Observations of HAA in a blood donor/recipient system in Thailand as it appeared in the SEATO Medical Research Laboratory Annual Progress Report, 1971-1972, pp. 140-155. The study concerns the development of post-transfusion hepatitis in persons receiving units of blood containing hepatitis-B antigen (HB-Ag, previously designated HAA). The donated units are identified and when transfused the unit is tested and the recipient categorized as either a recipient of positive or negative blood, the recipients of negative blood acting as a "control" population.

The objective of these studies is to determine the results of the transfusion of blood containing hepatitis B antigen to a population of Thais where the antigen is highly endemic.

Approximately 1000 more persons have been added to the study. The study population of persons followed more than 6 months has been expanded to over 125, and a similar number of controls have been followed for this period. In a number of cases, patients have now been followed for more than 12-18 months. New patients have not been admitted to the study since November 1972 and the input of patient data is essentially complete. Presently, improved techniques for HB-Ag and HB-Ab are being developed and will be used with the stored sera. Collation of the data is in progress. The data should provide information on the following important areas of consideration:

- a. rate of development of HB-Ag after receiving a unit containing the antigen;
- b. rate of development of clinical and subclinical hepatitis following transfusion of a unit containing the antigen;
- c. persistence of developed HB-Ag;
- d. time sequence of the development of HB-Ag and of hepatitis;
- e. time sequence of SGOT rise and SGPT rise and an indication of the relative sensitivity and specificity of these tests for evaluation of liver disease;
- f. the significance of prior possession of antigen or antibody with the subsequent receiving of a unit of blood containing HB-Ag or HB-Ab;

g. some indication of the relationship between HB-Ag and liver disease;

h. an indication of the prevalence of HB-Ag and HB-Ab among a population of ill persons requiring transfusion;

i. blood group differences in HB-Ag acquisition.

This study has just completed its second year of followup. To date, 2840 persons have received transfusions and been followed in the study. Approximately 300 persons have been followed for 6 months or longer. Early data indicate that overt hepatitis following HB-Ag transfusion is a surprisingly rare occurrence in the study population.

4. Prevalence of Hepatitis B Antigen Carriers in American Personnel Entering and Leaving the Republic of Vietnam.

A study was conducted to determine whether the prevalence of hepatitis B antigen (HB-Ag) among American personnel increases with exposure to indigenous tropical populations with a high carrier rate of antigen and endemic hepaticis.

The prevalence of HB-Ag has been determined for a number of defined populations. Chronic HB antigenemia in temperate zones ranges between 0.1 and 0.5% while tropical populations have prevalences between 5 and 10%. Prevalence rates in the Republic of Vietnam have been found to be approximately 10%.

Studies of HB-Ag in tropical populations indicate that children may be exposed and become carriers at an early age. It is not clear whether the early acquisition of antigen in these populations is related to differences in immune mechanisms due to infancy, to the prevalence of antigen in the environment and/or to other factors such as a genetic predisposition to antigen carriage.

In addition to studying the prevalence of HB-Ag in Americans leaving Vietnam, a study of the subtypes of these individuals was undertaken in an attempt to discover the origin of these infections. Subtype adr, the predominant subtype found in Southeast Asia, is rare in temperate climates.

Sera were collected from 1293 men arriving in Vietnam during October and November 1970 and from 1072 men leaving Vietnam in November and December 1970. Only those personnel who had been in the Republic of Vietnam for 10 months or more were studied at departure.

Sera were tested for the presence of HB-Ag by immunoelectroosmophoresis (IEOP). Complement fixation (CF) titers were determined

on those sera found positive. Antigens were subtyped by agar gel diffusion, using rabbit antisera kindly provided by LTC William H. Bancroft of Walter Reed Army Institute of Research. The method of subtyping is described elsewhere in this report.

Of the 1293 sera obtained from men arriving in Vietnam, 11 or 0.85% contained HB-Ag by IEOP. Five of 1072 or 0.5% of the men leaving after a tour of 10 months or greater had antigenemia (see Table 1). The men arriving in the tropics formed two radically different groups. The first group of 1004, who were entering Vietnam for their initial overseas tour, (mostly low ranking enlisted men) showed a prevalence of HB-Ag of 0.4%. The remaining 289, who were returning to Vietnam for subsequent tours (an older and higher ranking group) had a carrier rate of 2.4%. It is of note that at least one of these individuals returning to Vietnam was noted to have marks of repeated injections on both arms.

Subtyping of antigen was undertaken on the 16 positive specimens and subtypes were defined in 14 (see Table 2). Twelve of these had complement fixation titers of antigen greater than or equal to 1:16 and required no concentration for typing. The other two sera required concentration with lyphogel before clear precipitin spurs indicating subtype were demonstrable. All sera showed the presence of the group reactive a antigen. There were four ay subtypes, all of which were ayw. Ad subtypes accounted for eight of the remaining ten; two were adw and four were adr. In one case a third antigen beyond ad could not be determined. On repeated testing, two antigens appeared to have four determinants. In both instances the a and the w component were clearly defined. However, the d and the y determinants, which previously had been thought to be mutually exclusive, were both present. Selective adsorption of these sera with antibody containing one of these determinants appeared to absorb out the other indicating that both determinants were present on the same particle.

Sera from three groups of men were sampled for the presence of HB-Ag. The cohort entering the tropics for an initial tour showed a prevalence of antigen which was high but within the range found in populations in the United States. Those leaving the tropics showed no increase in the percentage of men carrying HB-Ag despite considerable mixing with the local population. The degree of mixing can be partially estimated on the basis of a venereal disease incidence of 20% among these men during their tours. The cohort returning to the tropics for subsequent service showed a prevalence of HB-Ag six times that of those entering the tropics for their initial tour. The reason for this markedly increased prevalence of antigen carriers is unknown.

Subtyping of the detected antigens showed no predominance of any one subtype in any of the three groups of men (see Table 2). Adw

Table 1. Prevalence of Hepatitis B Antigen among US Military Personnel in the Republic of Vietnam

	# Tested	# Positive	% Positive
<u>Inprocessing</u>			
Initial Tour	1004	4	0.40%
Subsequent Tour	289	7	2.42%
Total Inprocessing	1293	11	0.85%
<u>Out Processing</u>	1072	5	0.47%
Total Tested	2365	16	0.68%

Table 2. Hepatitis B Antigen Positive Subjects among U.S. Military Personnel in the Republic of Vietnam

	Inprocessing											Out Processing				
	Initial Tour				Subsequent Tour											
Age	20	20	25	25	31	29	22	25	38	21	23	20	26	30	21	21
Rank	E3	E2	SSG	E3	E6	E6	E6	E6	E6	E5	E6	E4	E5	E5	E5	E4
Subtype	-	adw	adw	adr	ad(?)	adr	adr	ayw	ayw	ayw	adyw	adr	ayw	-	adyw	ad

and adr were both found in men entering Vietnam for the first time. Ayw was found in those subjects returning for a subsequent tour as well as those leaving the tropics. This may indicate that they had acquired their antigen in the United States as ayw is a rare subtype in Southeast Asian populations. It is of interest that both of the adyw subtypes reported above were found following Southeast Asian tours. Further investigation of these antigens is underway.

5. Subtyping of Hepatitis B Antigens in Indigenous and Foreign Populations in Southeast Asia.

The objective of this study is to develop the capability of subtyping hepatitis B antigen (HB-Ag) by the agar gel diffusion and other methods, and to use these methods to investigate the subtypes of HB-Ag found in Southeast Asia in both indigenous and foreign populations.

The heterogeneity of HB-Ag was originally described by Blumberg and associates and has been further investigated by Le Bouvier and Bancroft, et al, among others. This antigenic heterogeneity has been investigated using the Ouchterlony agar gel diffusion technique. Essentially, three major sets of antigenic determinants have been described. The first is a single determinant, "a," which appears to be common to all antigens. The second is one of a pair of determinants y or d which have been considered to be mutually exclusive alleles, and the third also contained one of a pair of mutually exclusive alleles which have been labelled w and r. Thus antigens described by these means are ayw, adw, adr and most recently ayr.

Observations as to distribution have shown geographical areas of prevalence of these subtypes. In the Far East ad has predominated and adr has been the major subtype detected. Recent investigation has also demonstrated the ayr subtype in samples originating from the Far East and it has been suggested that this subtype might emerge through mutation or recombination of separately infecting viral genomes. The unique opportunity in Thailand of studying both indigenous and foreign populations and the admixture of these two led to an effort to perfect methods of subtyping hepatitis B antigen in Thailand.

Sera: Over the past several years serum samples have been acquired from a number of different U.S. and Thai groups for both point prevalence studies of HB-Ag in populations and for identification of HB-Ag positive hepatitis. These samples have been tested for HB-Ag by several different methods (see elsewhere in this report) and those that were found to be positive were submitted for subtyping by the agar gel diffusion method.

Immunodiffusion Test: Alcohol-cleaned 1 x 3 inch microscope slides

were flooded with 3.0 ml of hot 0.8% agarose (Seakem) in 0.01 M tris EDTA buffer, pH 7.6. The agarose was allowed to solidify and then a pair of 7-well patterns were punched into it, forming wells which were 2 millimeters in diameter and 5 millimeters apart arranged in a hexagonal pattern around the central seventh well. The slides were stored in a moist chamber until used.

Sera to be tested were concentrated with Lyphogel. Concentration from five to ten fold was accomplished by placing 0.3 ml of serum in a tube with two granules of Lyphogel and incubating at room temperature for two hours. Concentration was helpful for serum in which antigen titered <1:16 by complement fixation. Reference antisera adw and adr were obtained from blood donors at the Phra Mongkut Klao Hospital in Bangkok. Reference antigen ayw and rabbit antisera against all three subtypes were kindly provided by LTC William H. Bancroft of the Department of Virus Diseases, Walter Reed Army Institute of Research. The sera were arranged on the slides placing each unknown serum next to a reference antigen so that lines of identity or partial identity could be easily detected. One of the antisera was placed in the central well.

Slides were incubated in a moist chamber at 37°C for 24 hours and then at 21°C for a further 48 hours or more. Slides were read at 24 hour intervals. Results were read as complete identity when test sera and reference antigen produced precipitation lines which fused without spurs. The presence of spurs was read as partial identity.

Table 1 shows the results of subtyping of antigens found in both American and Thai populations. Out of a total of 198 antigens detected in all groups, subtyping was possible in 109 or 55%. In 14 of these (13%) only a and d determinants could be detected; w and r determinants could not be defined. In 93, however, three sets of determinants were identified. Among these, ayw made up 23%, adw made up 10% and adr made up 67%. No ayr subtypes were found among American or Thai sera in this study. On repeated testing, two antigens from the U.S. troops in the Republic of Vietnam appeared to have four determinants. They clearly contained the common "a" determinant and the "w" determinant. The d and the y determinant, which previously had been thought to be mutually exclusive, were both present (see elsewhere in this report).

These data can be analyzed in two ways. First, subtypes obtained from hepatitis patients may be compared with subtypes obtained from carriers detected on point prevalence studies. It is of note that the percentage of subtypeable antigens in those subjects with hepatitis was much lower than those detected in point prevalence studies. This reflects largely the titer of the antigen in the serum, which is generally higher in carriers than in individuals suffering from disease.

Table 1 HB-Ag Subtypes of Thai and American Populations in Southeast Asia

Population	Number Studied	HB-Ag Positive %	With Typable Antigens	With ADR	With AD'V	With AYW	With AD	With Other Patterns
Thai lower socioeconomic Group	687	55(8)	** 40(73)	*** 32(8)	*** 5(12)	0	3(0.8)	
Thai Blood Donors	100	8(8)	8(100)	8(100)	0	0	0	
Thai Adult Hepatitis	113	⁴⁰ 51(45)	**** 11(28)	11(100)	0	0	0	
Thai Total	900	114(13)	59(52)	51(86)	5(8)	0	3(5)	
US Troop in VN	2365	16(0.6)	16(88)	4(28)	2(14)	4(28)	2(14)	****
US Hepatitis	174	68(39)	36(53)	7(19)	2(6)	18(51)	9(25)	
Total US	2539	84(33)	50(60)	11(22)	4(8)	22(44)	13(26)	
Grand Total	3439	198(6)	109(55)	62(57)	9(8)	22(20)	14(13)	2(2)

* IEOP positive

** Number subtyped (% of total positive)

***** adyw

*** Number of subtype (% of total subtyped)

**** 20 were submitted to subtype

Second, a comparison of subtypes obtained from Thai and Americans may be made. The total number of Thai subjects studied was 900 of which 114 or 13% were positive by IEOP. Of these, subtypes were identified in 59 or 52%, and in all of these the subtype was adr. In 3 of these or 5% no further determinants could be detected, however, 51 or 86% of the subtypable antigens were adr and 5 or 8% were adw. There were no ayw subtypes found among the Thai population studied, confirming the observations of Bancroft, et al, on smaller numbers of sera of a predominance of adr in Thailand. The adw subtypes were found in the point prevalence study of a lower socioeconomic Bangkok subdivision (see Annual Report, 1971-1972, pp. 129-139) and were found in three families, two in each of two families, and one in a third family. In three additional carriers in these families subtyping was not accomplished. All of the carriers in these three families in which subtypes could be identified were adw. This finding tends to support either intrafamilial spread of hepatitis B antigen or a genetic predisposition of families to certain subtypes.

The exclusive subtype detected among Thai adult populations with HB-Ag antigen positive hepatitis presenting at Phra Mongkut Klao Army Hospital was adr. Among this group of 113 individuals, hepatitis, b both positive and negative for HB-Ag, occurs 2-4 times as commonly in the decade between 20-30 as in any other age group (Annual Report, 1971-1972, pp. 117-120). This suggests that further investigation might show considerable cross-infection with hepatitis among Thai military personnel similar to that seen with adenovirus respiratory disease in United States military recruits.

In the American groups on the other hand, 2539 individuals were studied of which 84 or 3% were positive by IEOP for antigens and 59% of these or 50 were subtypable. The point prevalence study of HB-Ag in United States military personnel in the Republic of Vietnam has been reported elsewhere in this report. Little can be determined from the distribution of subtypes in this population because of the small number of carriers detected and the varied background of the men sampled. It is of note, however, that the two antigens subtyped as "adyw" were found in men who had spent at least one year in the Republic of Vietnam.

The subtyping of antigens obtained from Americans with hepatitis in the Republic of Vietnam is more revealing. 36 or 53% of 68 antigens positive by IEOP were subtypable. Of these, 18 or 50% were ayw. The remaining 18 were adr with 7 adr, 2 adw and 9 in which only "a" and "d" could be detected. Although we have not yet investigated the subtypes found among the Vietnamese population, it is highly probable that, like the Thais, the subtypes are predominantly adr. If this assumption is correct, the high proportion of ayw subtypes detected in HB-Ag positive disease among Americans in the Republic of Vietnam would indicate considerable cross-infection among American

military populations in Vietnam as opposed to acquisition of antigen from the indigenous population. Nine of the antigens isolated from these American hepatitis cases were not fully typed. Investigation of these specimens is continuing to determine whether these may not also show adyw or adyr subtypes.

In summary, HB-Ag subtyping by the agar gel diffusion method has been established in this laboratory. Results of early testing are shown in Table 1. Further investigations of Thai and American hepatitis B antigen positive subjects may shed light on transmission of hepatitis among military populations in Southeast Asia.

G. PROTEIN-CALCRIE MALNUTRITION

1. Mechanisms of Defective Delayed Cutaneous Hypersensitivity in Children with Protein-Calorie Malnutrition.

Cell-mediated immunity (CMI) plays an important role in determining the outcome of infections characterized by intracellular parasitism. Included among these infections are tuberculosis, moniliasis, herpes simplex, chicken pox, and measles, and these pathogens may produce unusually severe morbidity and mortality in malnourished individuals. Several investigators have recently reported defective CMI in African and Indian children with protein-calorie malnutrition (PCM). The possibility thus arises that a defective CMI response may be one immunological mechanism accounting for the severity of these selected infections.

One test commonly employed for assessing cell-mediated immunity is the delayed cutaneous hypersensitivity (DCH) reaction. According to current immunological concepts, DCH is a multistep reaction composed of at least three separate components (Figure 1). The sensitization (afferent) limb entails immunization of thymic-derived (T) lymphocytes against a macrophage-processed antigen. The recognition (efferent) limb is characterized by lymphokine production by sensitized T-lymphocytes after they recognize and interact with the antigen deposited in the skin. The inflammatory reaction, probably induced by lymphokines released at the skin site, is ultimately read as a positive DCH skin test. An intact inflammatory response is thus required for full expression of DCH, but it is not immunologically specific in that many irritants other than lymphokines can induce it. A defect in any one of the three components of DCH depicted in Figure 1 may account for the impaired skin test responses previously reported in PCM patients. The present study was designed to elucidate which of these three mechanisms was impaired in children with PCM and to measure immunological recovery during hospitalization and treatment.

Malnourished Patients: The patients, 1 to 5 years of age, were

admitted to the research ward of the Anemia and Malnutrition Research Center in Chiangmai, Thailand, where they remained throughout the 70-day study period. On admission the patients were diagnosed as having marasmus (M), marasmus-kwashiorkor (M-K), or kwashiorkor (K), using accepted criteria. Children admitted to the study had primary malnutrition and weighed between 3.0 and 12.0 kg. All patients were treated for fluid and electrolyte imbalance during the first seven hospital days and received supplemental vitamins and minerals. Virtually all patients on admission had bacterial infections and received appropriate antibiotic therapy until the infections cleared. Children were placed on gradually increasing calorie and protein milk-base formula diets, to a maximum intake of 175 calories - 4 gm protein/kg/day, which was started by hospital day 30 for all patients. Nutritional repair was judged complete when the clinical stigmata of PCM had disappeared, and the abnormal laboratory tests, such as low serum albumin, had returned to normal levels. Virtually all patients in this study had clinically recovered by 4 to 8 weeks after admission.

Dinitrofluorobenzene Skin Tests: 2, 4-dinitrofluorobenzene (DNFB), a potent skin irritant and contact allergen, was diluted in 90% acetone. A sensitizing and inflammatory dose of 2000 ug was applied to the forearm, allowed to dry, and protected for 24 hours with an occlusive dressing. At 48 hours, the degree of non-specific inflammation at the DNFB contact site was graded as follows: negative = no reaction or mild induration and/or erythema; positive = induration, erythema, and vesicle or bulla formation.

At varying time intervals from 14 to 70 days after application of the 2000 ug DNFB sensitizing dose, DCH responses were elicited by applying 100 ug DNFB to the opposite forearm. The degree of inflammation at the 100 ug test dose site was graded 48 hours later. Presence of erythema, induration and vesiculation was considered skin test positive. No reaction or presence of only induration and/or erythema was considered skin test negative. These rigorous criteria for a positive skin test were selected in order to minimize possible confusion with the mild non-specific cutaneous inflammatory reactions occasionally produced by 100 ug DNFB.

Candida albicans Skin Tests: 0.1 ml of a 1:100 dilution of Candida albicans skin test antigen (Hollister-Stier Laboratories, Spokane, Washington) was injected intradermally into the forearm. Reactions measuring greater than 5 mm induration at 48 hours were considered positive.

Lymphocyte Counts: Peripheral blood lymphocyte counts, performed on the first 25 patients admitted to the study, were within normal limits.

Cutaneous Inflammatory Response: Patients, presumed not to be immune to DNFB, were exposed to a 2000 ug dose on admission; only 13% had

responded with a marked inflammatory reaction when measured two days after exposure (Table 1).

More patients had an inflammatory reaction when first tested on day 15 (during clinical recovery), while the largest percentage responded on day 56 (after clinical recovery). The difference in the percentage of positive inflammatory responses between day 1 and day 56 is statistically significant (chi-square = 9.4, $P < .01$).

Evaluation of the Sensitization Component: We investigated the status of the sensitization component of the DCH reaction by attempting to sensitize three groups of patients against 2000 μ g DNFB. These patients, exposed to DNFB for the first time either on day 1, 15, or 56, were all skin tested for possible sensitization on day 70. The results are shown in Table 2. The difference between the low proportion of patients sensitized on day 1 and the high proportion sensitized on day 56 was statistically significant (chi-square = 4.7, $P < .05$). The data also suggest that the impaired sensitizing component apparently existing on day 1 may not be repaired until after day 15. It was of interest to examine the relationship existing between DNFB inflammation and subsequent sensitization. Accordingly, these two reactions were examined in each of the 23 patients listed in Table 2, and the comparisons are shown in Table 3. Of the 10 patients who were inflammation positive, 7 were subsequently found to have been sensitized. A similar direct association exists for the negative reactors, so that of the 13 inflammatory negative patients, 10 failed to be sensitized. However, the numbers of patients in each group are small and the differences do not reach statistical significance (chi-square = 3.27, $.05 < P < .10$).

Evaluation of Immunological Recall: Recall in DCH is a response characterized by recognition and interaction of sensitized lymphocytes with specific intracutaneous antigen which leads to inflammation at the skin site (Fig. 1). Recall was tested with Candida albicans skin test antigen. We assumed most patients had been naturally immunized against candida prior to their illness. This assumption is based on the observation that 70% of well-nourished Thai children over the age of 1 year are candida skin test positive.

Only 14% of PCM children were candida skin test positive on admission (Table 4). Skin test negative patients were retested on day 29 and 70 and an increasing percentage had converted to positive on these two days. Repeated skin tests with candida antigen did not induce DCH to candida in seven well-nourished skin test negative children, thus indicating that the increasing number of positive skin tests during hospitalization was not simply due to immunization by skin test antigen. The differences in numbers of skin test positive responders on days 29 and 70 compared to day 1 was statistically significant (McNemar test corrected for continuity; chi-square = 9.1, $P < .01$ for day 70).

Table 1. Cutaneous Inflammatory Response to 2000 μ g Dinitrofluorobenzene

Day Tested	No. of Patients	Skin Response		
		Positive	Negative	Percent Positive
1	30	4	26	13%
15	5	3	2	60%
56	8	6	2	75%

Table 2. Attempt to Sensitize PCM Patients against 2000 μ g Dinitrofluorobenzene

Day Sensitizing Dose Applied	No. of Patients	Skin Test Response*		
		Positive	Negative	Percent Positive
1	10	2	8	20%
15	5	1	4	20%
56	8	7	1	88%

* Skin test dose of 100 μ g DNFB applied on day 70 and read on day 72.

Table 3. Correlation of Cutaneous Inflammation and Sensitization
by 2000 μ g Dinitrofluorobenzene

Sensitized *	Number of Patients Who Were Inflammation+	
	Positive	Negative
Yes	7	3
No	3	10

* DNFB skin test either positive or negative on day 70.

+ Inflammatory response graded 2 days after application of 2000 μ g DNFB on hospital day 1, 15, or 56.

Table 4. Candida albicans Skin Test Recall Response

Day Tested	Number of Patients	Skin Test Response*		Accumulated Percent Positive
		Positive	Negative	
1	14	2	12	14%
29	12	8	4	72%
70	4	3	1	92%

* Skin tests read 2 days after 0.1 ml 1:100 antigen injected intradermally (positive = >5 mm induration).

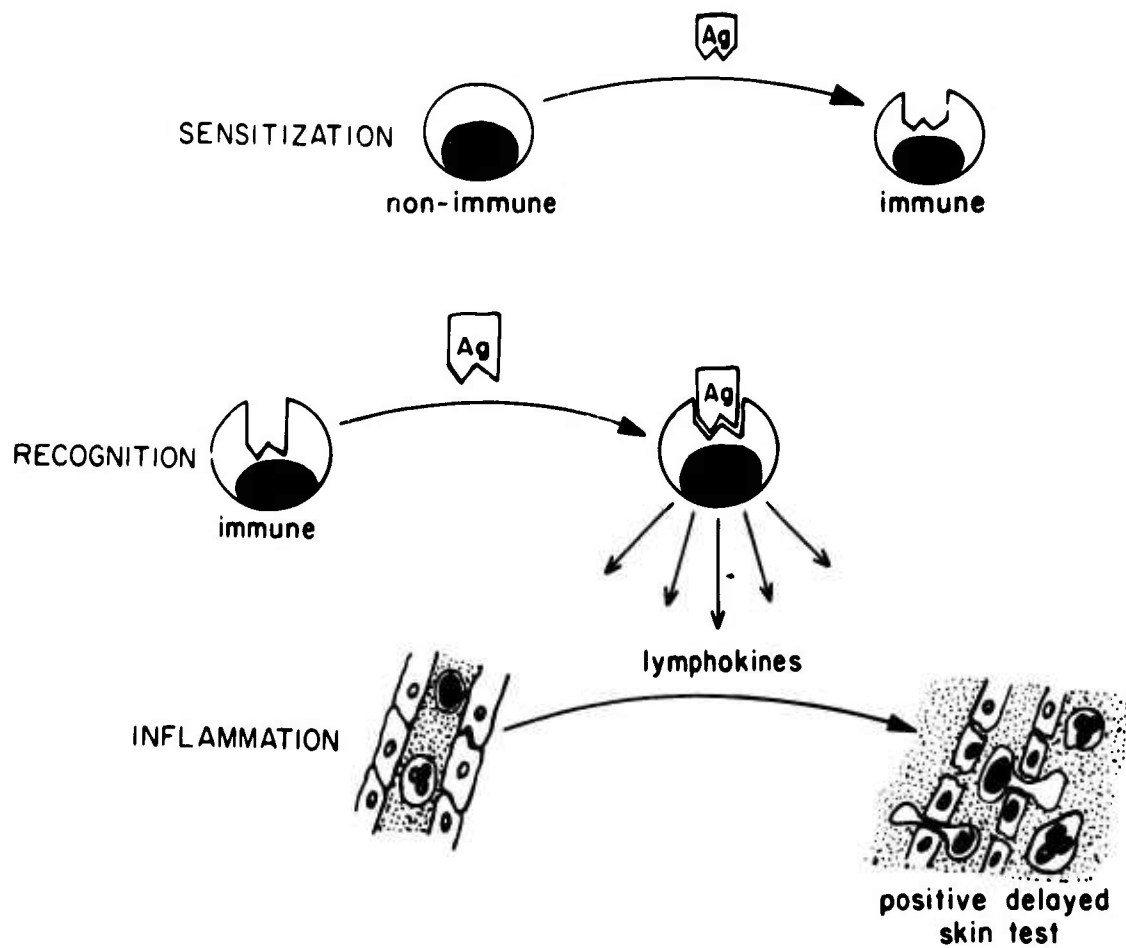


Figure 1. Schematic Drawing of Components of Delayed Cutaneous Hypersensitivity

We attempted to evaluate the three principal components of the delayed cutaneous hypersensitivity (DCH) response in children hospitalized with protein-calorie malnutrition. The lymphocyte sensitization component (afferent limb) and the cutaneous inflammatory reaction were evaluated with the contact allergen and skin irritant, dinitrofluorobenzene (DNFB); the immune lymphocyte recognition component (efferent limb) was tested with Candida albicans skin test antigen. The results indicated that 60 to 80% of PCM patients on admission had malfunction of both their afferent limb and their cutaneous inflammatory response. Two patients with intact inflammatory responses to DNFB on admission, but with negative candida skin tests, later displayed positive candida skin tests, suggesting that the efferent limb was defective on admission. Except for these two patients, the impaired inflammatory reaction precluded independent evaluation of the efferent limb in vivo. The DCH components were intact in most patients after nutritional repair, 1 to 2 months later.

2. Complement and C3 Proactivator Levels in Children with Protein-Calorie Malnutrition and the Effect of Dietary Treatment.

It is generally stated that malnutrition is associated with increased susceptibility to infection and that, in malnourished individuals, the infection is often more severe and recovery is slower than in well-nourished persons. Although association between protein-calorie malnutrition (PCM) and the increased susceptibility to infection has never been adequately proven by carefully controlled clinical studies, the weight of clinical experience by many investigators suggests that such a relationship does indeed exist. On the other hand, slow recovery from infection in PCM has been adequately documented, especially in experimental animals. It is difficult to design and control experiments in humans showing that malnourished individuals are more susceptible to infection; one can, however, look for changes in components known to be associated with defense mechanisms against infection, e.g., cell-mediated immune function, antibody and inflammatory responses, phagocytic and microbicidal activities of leukocytes, and the complement system.

The effect of PCM on the humoral immune response has been extensively investigated; the results are, however, inconclusive. While antibody response to some antigens was markedly depressed in PCM, the response to most antigens was unaffected. On the other hand, the cell-mediated immune function in a malnourished population is depressed somewhat. The hemolytic complement activity of the serum from malnourished children is also less than that of the well-fed children. Because a defect of the complement system is known to be associated with increased susceptibility to infection and malnourished children are prone to develop a gram-negative septicemia, impairment of the complement system in these children might be expected. The present study was therefore designed to examine the effect of protein-calorie

malnutrition on both the classical complement system and the newly discovered alternate C3-activating system. The serum levels of individual complement proteins, including C1q, C1s, C3, C4, C5, C6, C8 and C9, and of C3-proactivator (C3PA) of children with kwashiorkor and marasmus were determined on hospital admission and at intervals thereafter during dietary treatment. The results showed that, although the admission levels of these complement components and of C3PA were significantly lower than those of well-nourished children of the same age, most components rose to "normal" levels after appropriate dietary treatment.

Patients: The patients, age 1 to 5 years, were admitted to the research ward of the Anemia and Malnutrition Research Center in Chiangmai, Thailand, and remained in the hospital throughout the study period. On admission, the patients were clinically evaluated and classified as having marasmus or kwashiorkor according to the clinical criteria. Only children with primary malnutrition who weighed more than 3 kilograms but less than 12 kilograms were admitted to the study. All patients were evaluated on admission for evidence of infection, using clinical impression and the results of differential leukocyte counts, chest x-ray, and blood, urine and stool cultures. By these criteria, a majority of the 20 malnourished patients available for the present study were considered to be infected on admission. Those who were infected were placed on broad spectrum antibiotic therapy which included one or more of the following antibiotics depending on the site of infection: ampicillin, methicillin, gentamycin and cephalothin.

The patient's 71-day hospital course was divided into three main periods. During the stabilization period (days 1-7), all patients were given a gradually increasing caloric intake, from 25 calories- 1 g protein per kilogram per day at day 1 to 100 calories-1 g protein per kilogram per day at day 7. The stabilization period was followed by a 3-week study period (days 8-29) when the patients were randomly assigned to 1 of 4 dietary treatment schemes (Table 1). All patients again received the same diet (175 calories-4 g protein per kilogram per day) after hospital day 29. In addition to the above milk-base formula diets, a complete vitamin and mineral supplement was given starting on day 2.

Collections of specimens: Blood was collected on days 1, 8, 29, and 71 (venipuncture) and allowed to clot at room temperature. Serum was collected and immediately frozen at -20°C for a period of up to three months before analysis. In some cases, only plasma was available. Because both serum and plasma give identical results in the analysis for the complement and the C3PA levels by radial immunodiffusion, for ease of communication, they are referred to as serum throughout the remainder of this report.

Quantitative determination of individual complement proteins and C3-proactivator: The concentrations of C1q, C1s, C3, C4, C5, C6, C8, C9, and C3PA were measured by the radial immunodiffusion technique using immunoplates containing antiserum specific for individual complement components and for C3PA. The immunoplates and the reference standards were kindly supplied by Dr. H. J. Muller-Eberhard (Scripps Clinic and Research Foundation, La Jolla, California, U.S.A.). Seven microliters of reference standards and test specimens, appropriately diluted in a pH 8.0 isotonic buffer that contained 0.1M sodium chloride, 0.03M potassium phosphate, and 0.01 M trisodium ethylenediaminetetraacetate, were placed in the antigen wells. The plates were incubated at room temperature for 48-72 hours, and the precipitin rings were measured directly with a calibrated magnifier instead of from photographs as originally described. The reproducibility of the technique, as performed in this laboratory, was between 15 and 20%. All specimens were provided in coded form. The values reported for each specimen were the average of values determined at two different serum dilutions.

Complement and C3-proactivator levels in malnourished children: As shown in Figure 1, the mean concentrations of 7 of the 8 complement proteins (C4 excepted) and of C3PA in the serum obtained on day 1 from 10 marasmic and 10 kwashiorkor children were significantly lower than the means of samples from 19 normal children of similar age residing in the same area (P less than 0.05). The mean concentration of C4 was less than that of controls, but the difference was not statistically significant (P greater than 0.05). The 8 complement proteins did not appreciably increase during the initial 8 day stabilization period. However, on day 29, a marked increase in the level of all components except C9 was observed. The levels of C1q, C1s, C3, C4 and C5 reached maxima on days 29 or 71 which were significantly higher than the normal mean. It appeared that the relative rise in concentrations of the late-acting components, i.e., C5, C6, C8 and C9, was not as dramatic as that of the early-acting components, particularly C3 and C1s. The level of C9 did not return to normal during the 71-day period. The concentration of C3PA reached normal levels by day 29. The composite results shown in Figure 1 were next analyzed according to clinical groups and to the types of dietary treatment.

Comparison of complement and C3 proactivator levels in marasmic and kwashiorkor children: The admission levels of 7 of the 8 complement proteins (C4 excepted) and of C3PA in the 10 children with kwashiorkor were lower than the corresponding levels of the 10 marasmic children of the same age (Figure 2). The differences between the two groups were statistically significant at the 5% level only for C1q, C6, and C8. Of the 9 proteins analyzed, only the concentrations of C5, C6 and C3PA from the marasmic group were significantly lower than the "normal" controls (P less than 0.05), suggesting that malnourished children of the marasmic type were less severely affected by PCM. However, by day 8 the complement and the C3PA levels were similar in both clinical

groups and remained so throughout the study.

Effects of dietary treatment on the recovery of complement and of C3 proactivator levels: Because preliminary analysis of the data discussed above indicated that the children with marasmus and kwashiorkor responded similarly to dietary treatment, the data from these two groups were combined for analysis of the effects of the different dietary treatments. The 20 children were divided into four dietary groups as shown in Table 1.

The quantity of dietary protein markedly influenced the levels of complement proteins and C3PA (Figure 3). The concentrations of the complement proteins and C3PA of the high and low protein dietary groups were both low before institution of special dietary treatment on day 8. The stimulating effect of higher protein intake was apparent on day 29, three weeks after the start of the treatment diets; the children who received 4 g of protein per kilogram per day (group 4) had reached normal or above normal complement levels, whereas the children who received 1 g of protein per kilogram per day (group 3) had not only failed to increase their complement levels, but also suffered a further reduction of some components. Forty-one days after their diet was changed from 1 g to 4 g of protein per kilogram per day, the low protein group showed significant improvement in the complement levels (day 71). As shown in Figure 3, the concentrations of the seven complement proteins (C4 excepted) and of C3PA of both dietary groups were indistinguishable on day 71.

The effect of caloric intake on the recovery of the complement and C3PA levels was not as dramatic as that for protein (Figure 4). Although the number of cases was small, the children who received the high caloric diet (group 4) tended to respond better than those on the low caloric diet (group 2).

Because only two children comprised diet group 1, the changes after dietary treatment may not be representative of the group. It appeared however, that some improvement of the complement levels were achieved on a diet of 100 calories-1 g protein per kilogram per day. The recovery seemed to be slower and less complete than in the other groups.

Effect of infection on the complement profile in PCM children: Serum samples taken from two infected, malnourished children a few days before or on the day of death showed the complement and the C3PA levels to be markedly depressed (Table 2). All complement components and C3PA were noticeably lower than those from PCM children who recovered. Many components, in fact, dropped below the lower limits of sensitivity of the detection method.

Summary: The serum levels of complement proteins (C1q, C1s, C3, C4,

Table 1. Dietary treatment and clinical status of PCM children.

Dietary Group	Dietary Intake		No. Children		
	Calories/kg/day	G protein/kg/day	Marasmus	Kwashiorkor	Total
1	100	1	1	1	2
2	100	4	3	1	4
3	175	1	3	3	6
4	175	4	3	5	8

Table 2. Complement and C3PA levels in malnourished children who died during hospitalization.

Patient	Cause of death	Hospital Day	Per cent of normal							
			Clq	Cl _s	C3	C5	C6	C8	C9	C3PA
A	Sepsis	1	<18	30	12	40	32	<36	37	46
		3 (Immediately before death)	<18	30	10	37	28	<36	22	28
B	Sepsis	2 (One day before death)	<18	<18	5	<16	14	<36	<11	<13
PCM children (Mean values from 20 subjects who recovered)		1	84	69	63	79	59	75	55	61

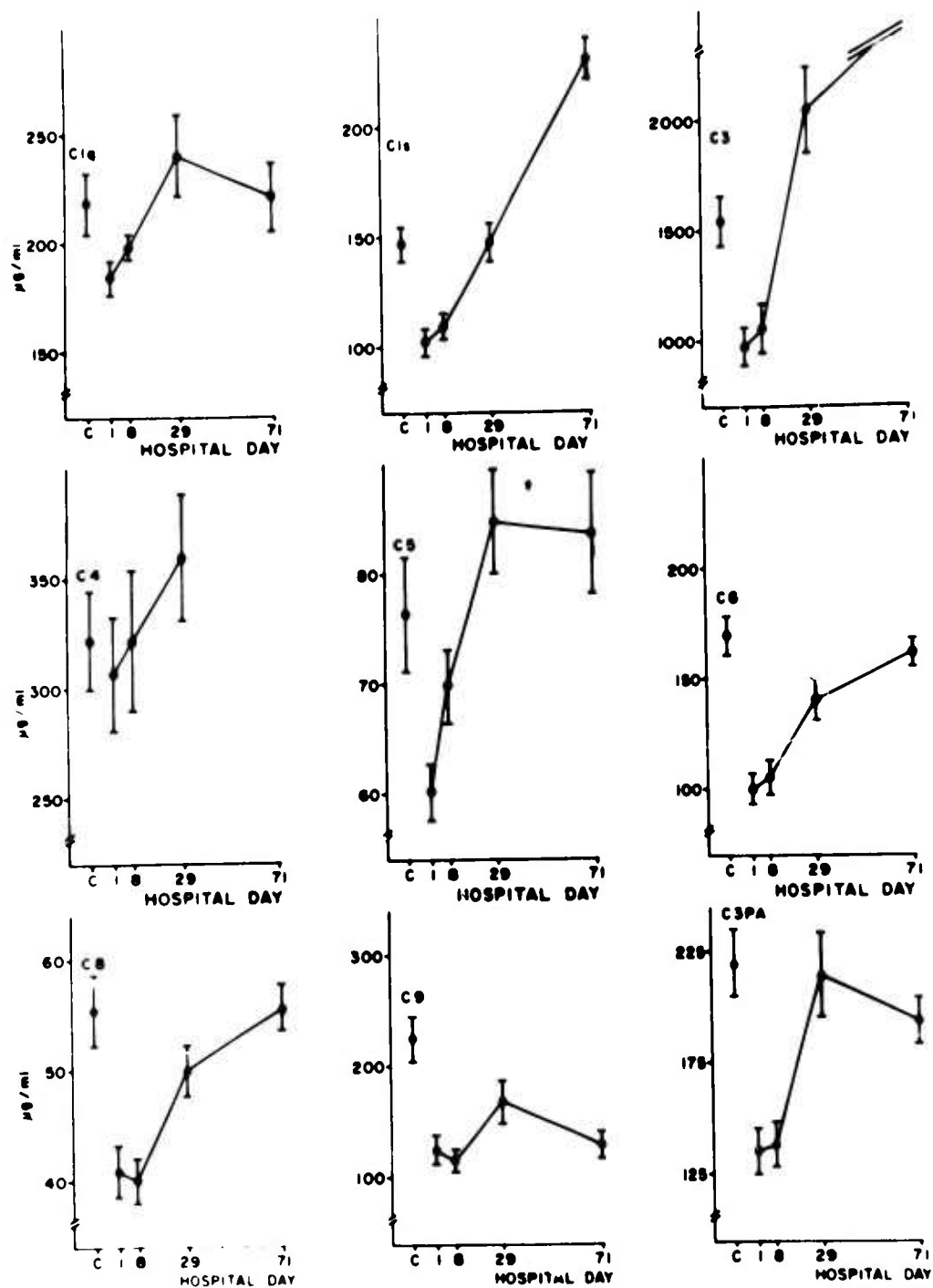


Figure 1. Mean Concentrations of Complement Proteins in Children with Protein Calorie Malnutrition

ADMISSION SERUM COMPLEMENT LEVELS IN MARASMUS AND KWASHIORKOR CHILDREN

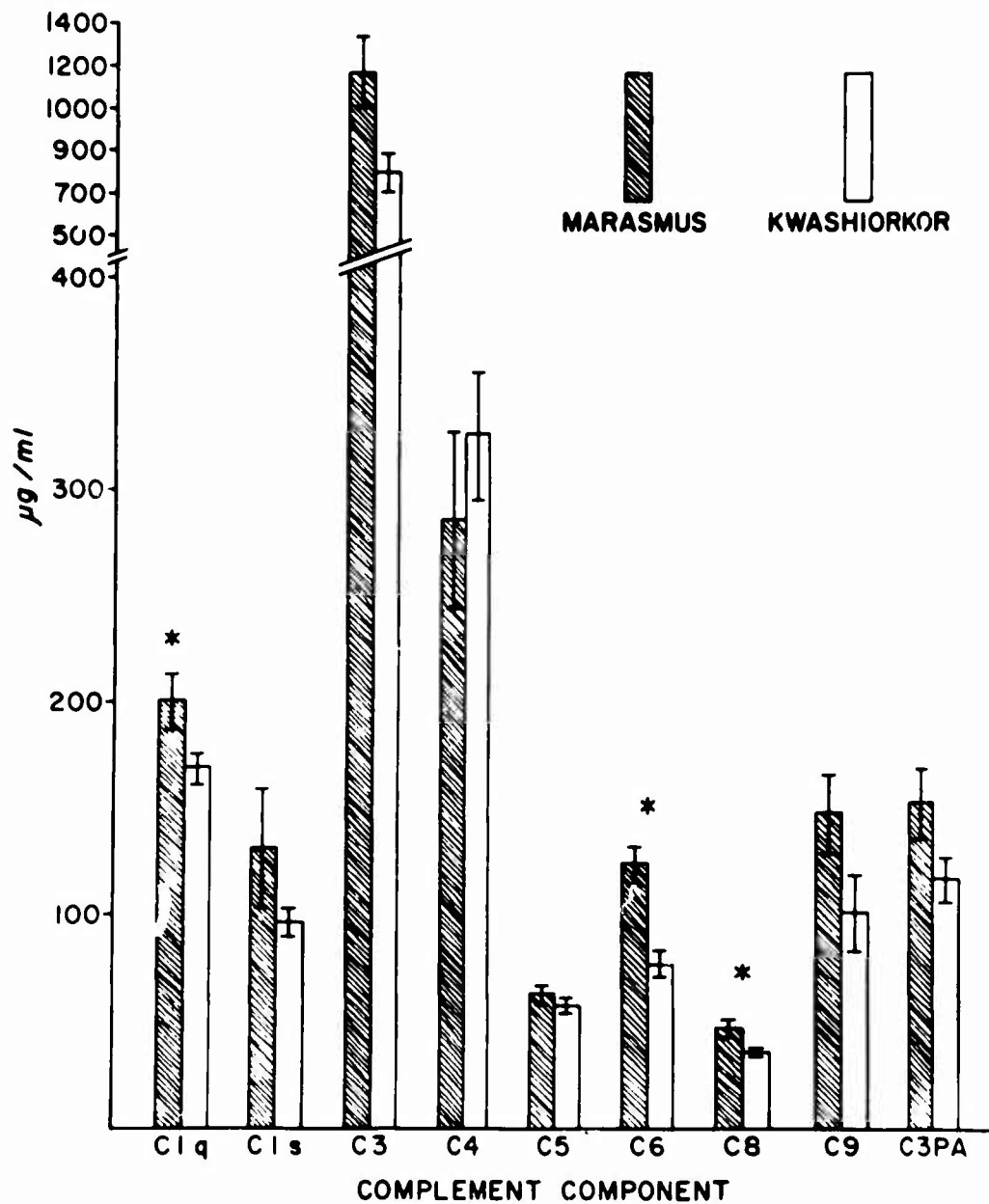


Figure 2. Mean Admission Concentrations of Complement Proteins in 10 Children with Kwashiorkor and Marasmus

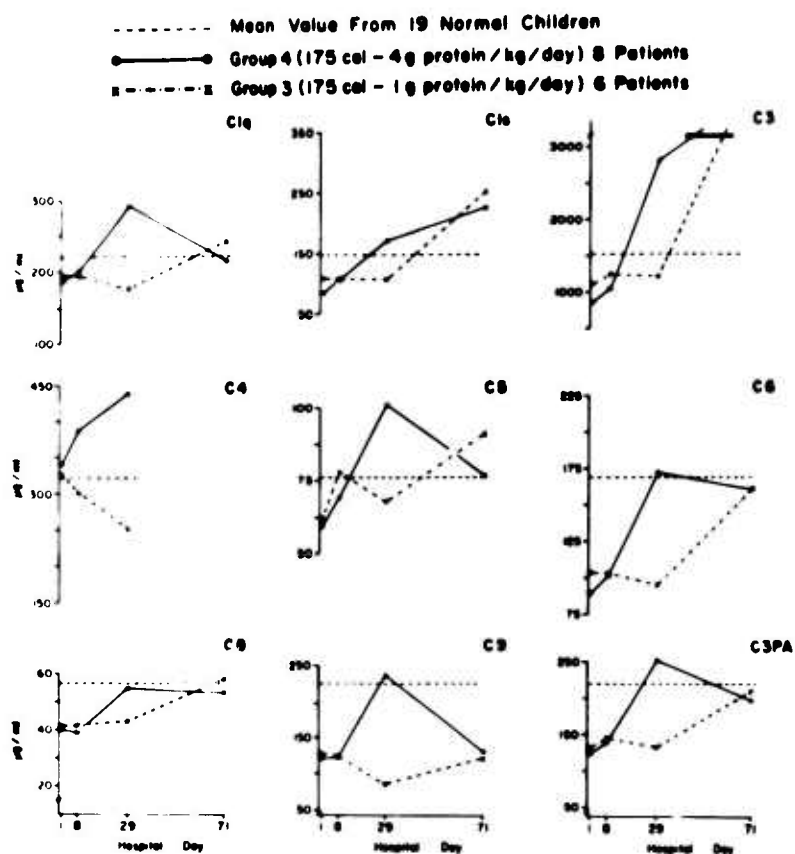


Figure 3. Effect of Amount of Dietary Protein on Serum Complement Protein Concentration

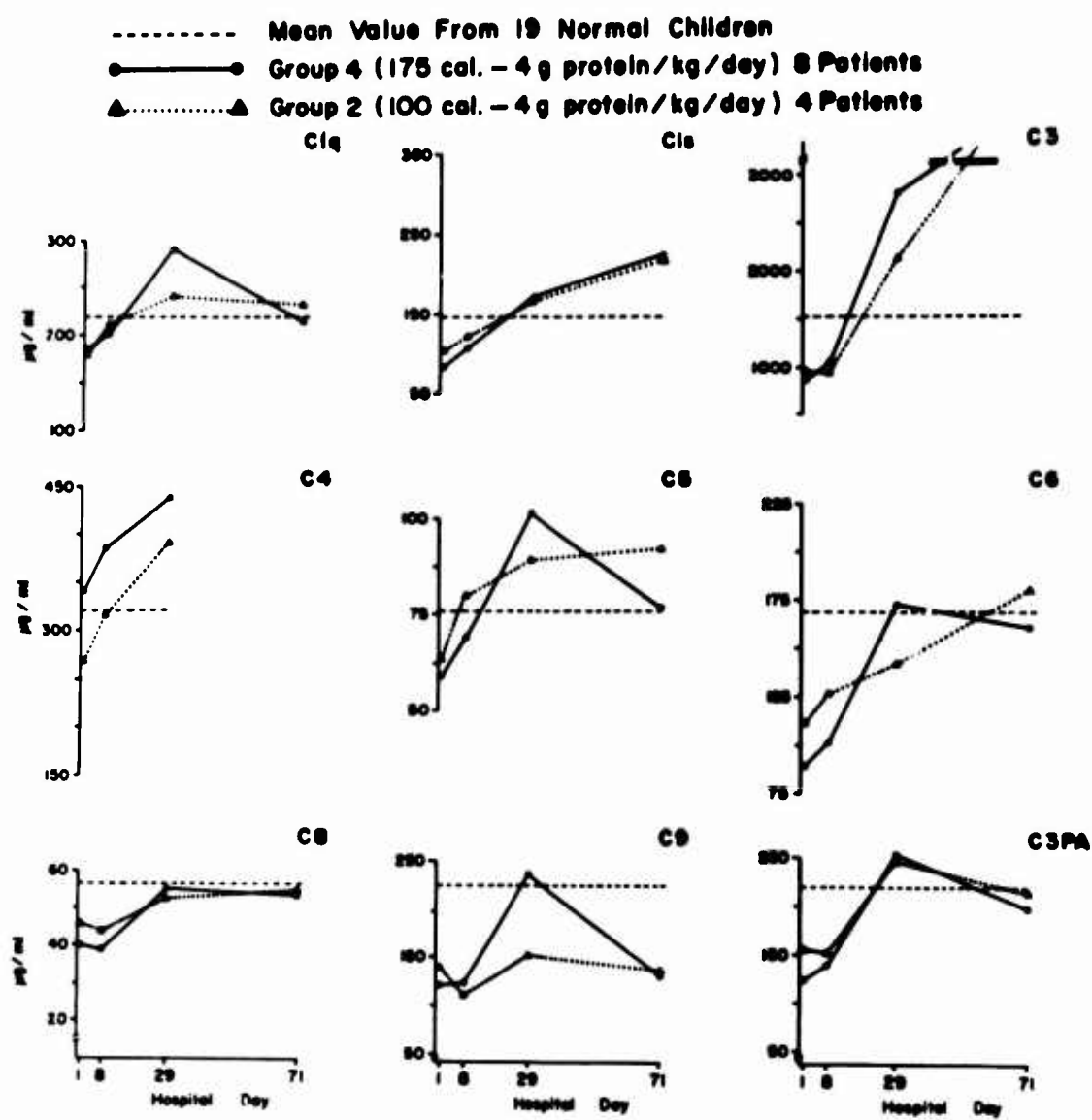


Figure 4. Effect of Caloric Intake on Recovery of Complement Protein Levels

C5, C6, C8 and C9) and of C3 proactivator in 20 children with protein-calorie malnutrition were compared with those of "normal" children of the same age residing in the same geographical area. These components were determined on admission and at intervals during different dietary treatment regimens. All components, except C4, were markedly lower in the malnourished children on admission than in the "normal" children, and the children with kwashiorkor were more severely affected than the children with marasmus. The quantity of dietary protein, and to a lesser extent the caloric intake, had a marked influence on the repair of the complement system.

3. Complement Activity in Protein-Calorie Malnutrition.

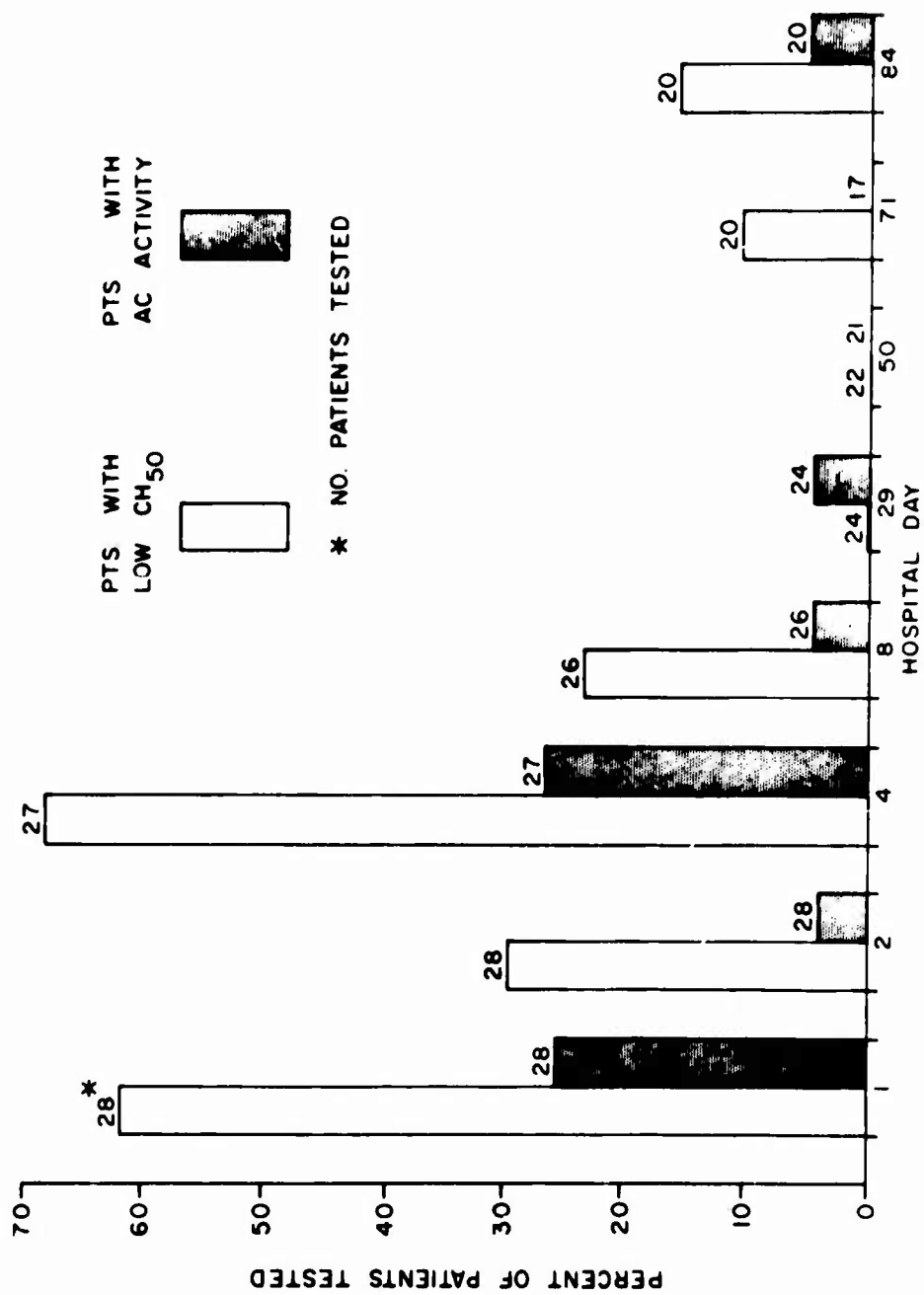
Several defective host defense mechanisms have been implicated by us and others as increasing the susceptibility of children with protein-calorie malnutrition (PCM) to infection. Recent studies described elsewhere in this Annual Report have indicated that complement protein concentrations are abnormally low in untreated PCM. In an effort to further evaluate the competence of the complement system, 28 children with PCM were evaluated for serum hemolytic complement (CH_{50}) activity and for anti-complementary (AC) activity during the acute phase of the disease and throughout recovery.

Children were studied on admission and on hospital days 2, 4, 8, 29, 71, and 84 for CH_{50} and AC activity. CH_{50} was measured using standard methods. AC activity was measured by mixing equal volumes of unheated patients' serum with standard serum of known CH_{50} titer. The CH_{50} activity of the mixed serums was then determined. Dilution controls were also run. The sera of 48 well-nourished Thai children mixed with a standard serum always resulted in a titer of 480 units/ml or more. If a serum (PCM) reduced the standard serum CH_{50} activity to below 480 units/ml after mixing the PCM serum was considered to contain AC activity. The 48 normal Thai children all had serum CH_{50} titers greater than 160 units/ml. Thus a CH_{50} titer was considered abnormally low in PCM if it measured less than 160 units per ml.

Serum CH_{50} activity was depressed in 60% of children with PCM on hospital admission (Figure 1). After nutritional repair there were fewer patients with depressed CH_{50} activity. Anticomplementary activity was present during the first week after admission in the plasma of 50% of children studied (Figure 1). After nutritional repair the number of patients showing AC activity decreased or disappeared. The patient group which received 4 gms of protein/kg/day appeared to have more rapid regeneration of CH_{50} activity than the group receiving 1 gm of protein/kg/day.

The low complement functional activity may reflect the low complement protein concentrations found in PCM. Like complement protein

Figure 1. SERUM HEMOLYTIC COMPLEMENT (CH_{50}) AND ANTI-COMPLEMENTARY (AC) ACTIVITY IN PCM PATIENTS DURING CONVALESCENCE



concentrations, the low hemolytic complement activity responds better to a high protein than to a low protein diet. Depressed CH₅₀ activity may be in part due to depressed complement synthesis in protein starvation and to the presence of AC activity in some sera. The nature of the AC activity (immune complexes or endotoxin) is being investigated. In addition, studies of C3 turnover with I¹²⁵-labelled C3 have been initiated in order to determine whether the depression in CH₅₀ activity may be secondary to increased consumption of complement in PCM.

4. Secretory and Serum IgA in Protein-Calorie Malnutrition.

Malnourished children suffer from an increased incidence and severity of infections. We have previously demonstrated an impairment of the cell-mediated immune function, of the inflammatory response, and of complement and C3-activating systems in children with protein-calorie malnutrition (PCM). (See this year's Annual Report).

Because children suffering PCM are prone to infection at the body surfaces, the local immune system was investigated in these children.

As shown in Figure 1, the total nasal-wash protein concentrations in untreated PCM patients and well-nourished Thai children were similar as were nasal-wash IgG and albumin levels. By contrast, the level of secretory IgA, expressed as percent of nasal wash protein, was significantly lower than that of control children ($P < .01$). The deficiency in secretory IgA failed to respond after 70 days of treatment with 175 calories and 4 gm protein per kilogram per day. There was significant improvement in other immune functions after this same dietary treatment. Late convalescent nasal washes are being examined now in order to determine how long the secretory IgA defect persists. Children with kwashiorkor and with marasmus were equally deficient in secretory IgA.

In contrast to secretory IgA, serum IgA concentrations were markedly elevated at the time of hospital admission in PCM patients. The mean serum IgA concentrations, however, decreased to the control level after dietary treatment.

The results suggest that (1) the systemic and the local IgA systems respond differently to dietary treatment of PCM and (2) the apparent deficiency of secretory IgA found in severe PCM could relate to the clinical observations that malnourished children seem prone to develop mucosal infections.

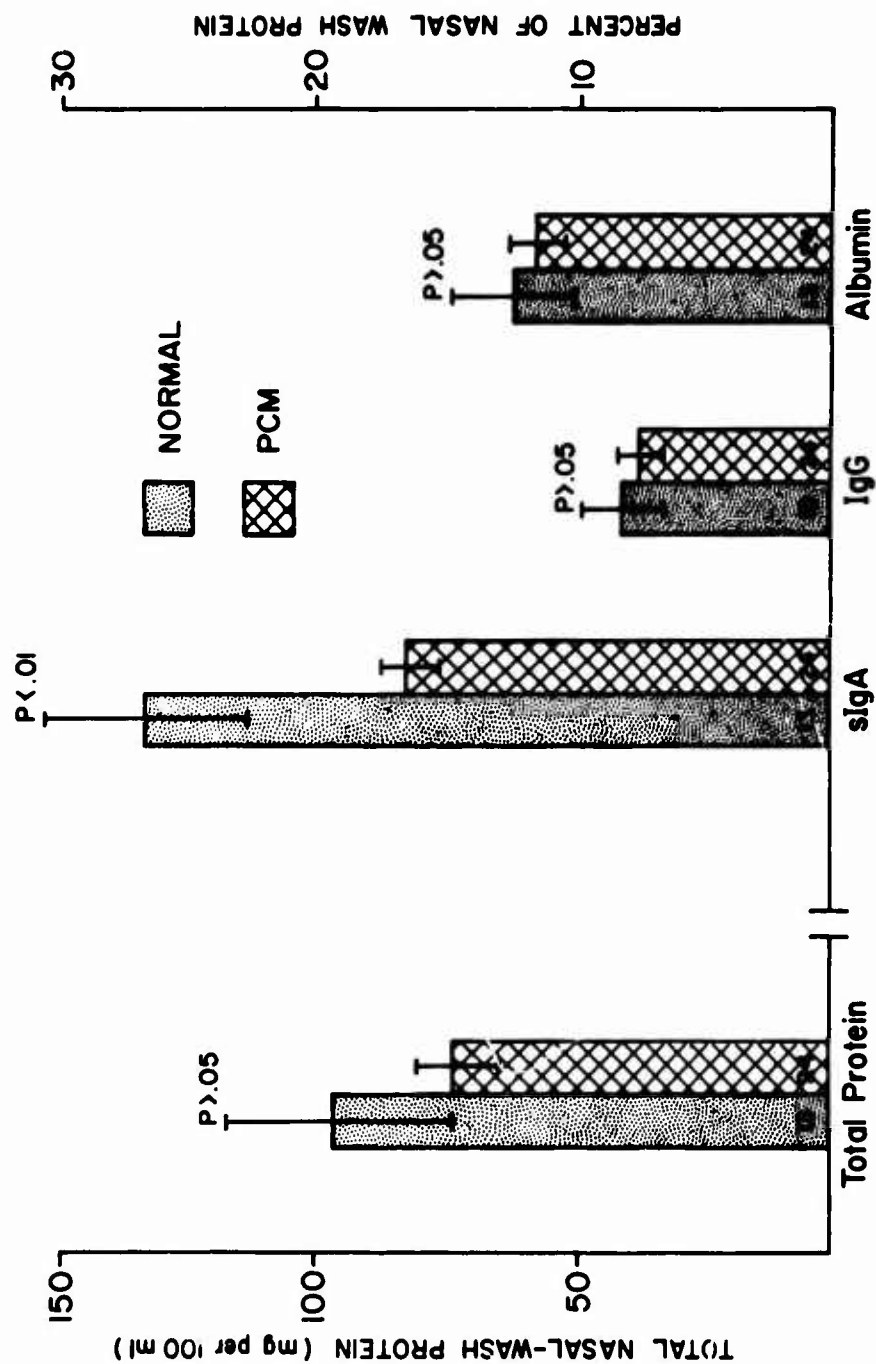


Figure 1.
Comparison of Nasal-Wash Protein Content of Well-Nourished and Protein-Calorie Malnourished Children. The mean values \pm one S.D. are indicated by the bars. The number of patients studied and probability levels are shown.

Project 3A062110A831 TROPICAL MEDICINE

Task 00, Tropical Medicine

Work Unit 002 Tropical and subtropical military medical research.

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Project 3A062110A 931 TROPICAL MEDICINE

Task 00, Tropical Medicine

Work Unit 002 ~~Tropical and subtropical military~~ medical research

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1. Mycotic Disease

This laboratory routinely conducts mycologic examination of clinical specimens submitted from various sources throughout Thailand. While no active mycological investigations were undertaken during the reporting period, a sufficient quantity of specimens were studied to insure an adequate exposure of our technicians to examination techniques. Such service to outside clinical facilities enables this laboratory to monitor mycotic diseases in Thailand, to maintain adequately trained technicians, and to insure media and reagent rotation. A stock of typical fungal cultures enables this laboratory to train medical technicians sent to us for such training

TABLE 1
Results of Examinations of Specimens Submitted
for Pathogenic Fungi
1 April 1972 - 1 March 1973

Source	Number of Specimens	Negative for Fungi	Non Pathogenic Fungi Isolated	Positive Cultures	Total/from US personnel
Body	54	13	11	<u>Candida albicans</u> <u>Trichophyton rubrum</u> <u>Microsporium gypseum</u> <u>Epidermophyton floccosum</u> <u>Trichophyton mentagrophytes</u>	4/2 * 21/0 2/1 2/0 * 1/0
Hair	3	2		<u>Trichophyton rubrum</u>	1/0
Ear	6	2	3	<u>Candida albicans</u>	1/1
Hand	5	1	1	<u>Candida albicans</u> <u>Trichophyton rubrum</u>	2/1 1/0
Nail	24	6	8	<u>Microsporium audouinii</u> <u>Candida albicans</u> <u>Trichophyton rubrum</u>	1/1 3/1 6/0
Skull	1	1			
Sputum	8	2	2	<u>Candida albicans</u>	4/3

TABLE 1 (Cont.)

Source	Number of Specimens	Negative for Fungi	Non Pathogenic Fungi Isolated	Positive Cultures	Total/ from US personnel
Foot	17	2	5	<u>Microsporium gypseum</u> <u>Epidermophyton floccosum</u> <u>Trichophyton rubrum</u> <u>Trichophyton mentagrophytes</u> <u>Candida albicans</u>	1/1 1/1 1/0 4/0 3/0
Leg	2			<u>Trichophyton rubrum</u> <u>Microsporium gypseum</u>	1/0 1/0
Eagle blood	3	1	2		
Gibbon lung	1			<u>Actinomyces bovis</u>	1/0
Air	11	1	10		
Vagina	3	1		<u>Candida albicans</u>	2/2

TABLE 1 (Cont.)

Source	Number of Specimens	Negative for Fungi	Non Pathogenic Fungi Isolated	Positive Cultures	Total/ from US personnel
Scrotal hair	3	3			
Pus	3		3		
Pleural fluid	1	1			
Cerebrospinal Fluid	7	7			
Thoracentesis	4	4			
Yeast for identification	10		2	<u>Candida albicans</u>	8/8
Sinus cavity	1			<u>Candida albicans</u>	1/1

* Two isolates from one specimen

from throughout Thailand.

During the past year, we have examined 122 specimens. The majority of these specimens came from the US Army Hospital in Bangkok, others from the Thai Army Hospital in Bangkok, and some 25 specimens were referred to this facility from hospitals throughout Thailand. From these 122 specimens, 42 pathogenic fungi were recovered, the majority (23) being Candida albicans. Table 1 presents the results of these examinations.

2. Gonorrhea in Asymptomatic Females.

While it has been demonstrated in several Western clinics that up to 8% of females seen at routine hospital visits harbour Neisseria gonorrhoeae infections, no such survey has ever been conducted in Thailand. Our study was designed to observe the incidence of clinical gonorrheal infections in asymptomatic Thai females.

Women from 20 years of age to 50 years of age were seen at Women's Hospital, Bangkok, and at the Thai Army Hospital, Bangkok. These patients were seen for routine Papanicolaou smears and for OB-GYN complaints other than cervicitis. Routine cervical cultures on prewarmed Thayer-Martin chocolate agar and on blood agar were made. Plates were immediately placed in CO₂ (candle) jars and incubated at 35-37°C within one hour. All plates were examined at SMRL the next day and routine identification procedures for N. gonorrhoeae were conducted.

One hundred sixty-four patients seen at Women's Hospital were examined and seven (4.2%) were found to be infected with N. gonorrhoeae. Table 1 presents this data by age group. Of 141 patients (military dependents) seen at the Thai Army Hospital, five (3.5%) were found to be infected.

This survey has brought to the attention of the investigators the need for routine N. gonorrhoeae cultures in asymptomatic females.

Table 1

Gonorrhea in Asymptomatic Females
Women's Hospital
Bangkok, Thailand.

Number of Patients	Age (y.s.)	Positive Cultures
35	20-30	5
79	31-40	2
50	41-50	0

3. The Efficacy of Trimethoprim/Sulphamethoxazole as an Oral Treatment of *Neisseria gonorrhoeae* Infection of Females.

An oral medication for the treatment of gonorrhea is desirable for several reasons; 1) the large amounts of penicillin required to effectively treat gonorrhea in South East Asia is often associated with a great deal of pain, 2) some individuals report a sensitivity to penicillin, and 3) as an alternative for treatment of penicillin resistant *Neisseria gonorrhoeae*. A real problem in this area of the world with oral medications is that the full regimen is often not completed by the patient. This may be due to an apparent abeyance of symptoms or the desire to share the medication with a friend or partner. We have therefore designed an efficacy protocol to evaluate the use of trimethoprim/sulphamethoxazole (co-trimoxazole) in increased quantities capable of being administered orally in the clinic for the treatment of gonorrhea in females.

Promiscuous Thai females reporting to a VD control clinic for routine examination were selected for this study. Those girls with positive (intracellular, gram negative diplococci) cervical smears were selected and assigned a two digit random number. Those with numbers totaling an even number were assigned to one protocol, those with odd sums, the other protocol.

One protocol (Y) called for immediate culture of the urethra, cervix, rectum, and vagina, acquisition of history and explanation of the study, and IM treatment with 4.0 mega units of penicillin G. Patients in group Y were requested to return to the clinic at 24 and 72 hours. At these visits, cultures were made of the same four sites to determine treatment cure or failure.

Protocol X called for cultures as above, history acquisition with specific questions regarding pregnancy, known blood dyscrasias, or any known sensitivity to drugs. Those patients with affirmative responses to the above three questions were eliminated from the study. After the history, the patients in group X were given 4 tablets, each containing 80 mg trimethoprim and 400 mg sulphamethoxazole. These tablets were administered with at least 300 ml of water. The patients were observed for any side-effects for 2 hours and then blood was obtained from the patients in group X to be used to monitor haemoglobin, erythrocyte count, leukocyte count, hematocrit, and cell morphology. At this time an additional 4 tablets of co-trimoxazole were again carefully administered. These patients

TABLE 1
**Results of Cultures of Neisseria gonorrhoeae in Co-trimoxazole
Treated Patients and Patients Receiving 4.0 mega units
of Penicillin G**

Treatment Regimen	(No. of Patients)	Initial visit	Second visit	Third visit
X	(13)	+	-	-
Y	(23)	+	-	-
X	(6)	+	+	-
Y	(1)	+	+	-
X	(14)	+	+	+
Y	(4)	+	+	+
X	(3)	+	-	+
Y	(5)	+	-	+

X = 12 tabs of co-trimoxazole in 24 hours

Y = 4.0 mega units penicillin G

were told to return to the clinic in 24 hours. The next day cultures were made from the 4 sites indicated above, blood for CBC was obtained, and another 4 tablets of co-trimoxazole were administered. The patients were requested to return the next day (72 hours after initial treatment) for final cultures. At each visit, the patient was examined and asked questions regarding side-effects.

To date, we have placed 106 females in this study. 72 have returned for the second and third visit with 3 of these excluded because their initial cultures were negative for N. gonorrhoeae. One of these had a positive culture on the third visit but was still excluded because we considered that culture the possible result of a new exposure.

With a positive culture from any one of the four culture sites on the third visit as our definition of treatment failure, we found that co-trimoxazole had a cure rate of 52.7% while 4.0 mega units of penicillin G had a cure rate of 66.6%. Table 1 presents this data. Chi square test reveals that with this population, the difference in cure rate is not significant. We have not observed any blood dyscrasias due to either of these two drug regimens. Four patients receiving co-trimoxazole complained of headache, 2 of headache and nausea, and another 2 complained of vomiting within 1-2 hours after taking the second series of tablets. Three patients being treated with 4.0 mega units of penicillin G had complaints, with 2 fainting after the injection and 1 reporting a rash within an hour after receiving medication.

It is evident from our data that the efficacy of co-trimoxazole cannot be evaluated with this sample size. We are continuing with this study and will increase the sample size.

4. Penicillinase Activity of Staphylococcus sp. Associated with Neisseria gonorrhoeae Infections.

Experiments have been conducted to determine the frequency of penicillinase producing Staphylococcus sp. found in association with gonococcal urethritis.

Penicillin is still the drug of choice for the treatment of gonorrhea in Thailand. Treatment failures or disease recurrences continue to appear, however, when up to 4.8 mega units of penicillin G are administered as is reported elsewhere in this report.

While the reasons for such treatment failures probably include impaired antibiotic absorption and the emergence of resistant N. gonorrhoeae, the possible presence of antibiotic neutralizing substances in close association with the infection should also be considered. In view of the widespread use of non-prescribed penicillin in this part of the world, we became especially concerned with penicillin neutralizing enzymes (penicillinase) produced by some mutants of staphylococci.

Staphylococcus sp. were obtained from blood agar plates inoculated with urethral exudate of male patients seen at a venereal disease clinic. Patients were diagnosed as positive for N. gonorrhoeae when intracellular gram negative diplococci were observed in smears of urethral exudate. Penicillinase activity was determined by the methods of Workman and Farrar⁽¹⁾ after the isolates had been identified by biochemical means.

Of the 104 isolates of Staphylococcus sp. isolated from 79 cases of gonorrhea and from 25 cases of non-gonococcal urethritis, 82 (78.8%) were found to be producers of penicillinase. Table 1 presents this data.

Table 1

Penicillinase Producing Staphylococcus
in Male Urethritis

Staphylococcus sp.	Gonococcal Urethritis	Non-gonococcal Urethritis
Penicillinase positive	64	18
Penicillinase negative	15	7

The collection of additional data is continuing. Experiments with mixed cultures of N. gonorrhoeae and penicillinase producing Staphylococcus sp. are being conducted to determine alterations in penicillin minimum inhibitory concentrations. Additional studies involving in vivo testing of such enzymes in rabbits are being undertaken.

5. Diarrheal Diseases.

This laboratory continues to serve as a reference laboratory for complete identification of enteric bacteria. Specimens (including clinical materials and pure cultures) are sent to this facility for complete biochemical and serologic testing. Such a service insures complete training of technicians at this facility and enables students from other medical facilities to train here. This laboratory monitors the occurrence of enteric pathogenic bacteria in diarrheal diseases in Thailand.

Of the 1610 specimens examined during the past year, some 382 were from infants being treated at Childrens Hospital, Bangkok. 517 specimens were from Thai military and dependent patients being examined at the Thai Army Hospital in Bangkok, while 113 came from US Peace Corps volunteers at stations throughout Thailand. Tables 1-5 report the findings of our examinations for enteric pathogens.

TABLE 1
Enterobacteriaceae Isolated from Patients with Acute Diarrhea in Thailand
3 April 1972 - 30 March 1973

Month	Thai National					American National						
	No. of Specimens	No. of Patients	Salmonella	Shigella	E. coli	Vibrio parahaemolyticus	No. of Specimens	No. of Patients	Salmonella	Shigella	E. coli	Vibrio parahaemolyticus
Apr	34	32	1	2	11	2	10	10	-	-	1	4
May	83	80	2	2	20	2	33	31	-	4	-	4
Jun	88	83	7	3	22	3	17	15	2	1	-	-
Jul	75	69	6	4	15	-	9	9	1	1	-	-
Aug	89	87	6	5	19	-	32	32	-	12	-	-
Sep	111	99	13	7	20	1	16	16	-	-	-	1
Oct	108	103	8	9	23	-	6	6	-	2	-	3
Nov	114	114	5	17	11	-	19	18	-	1	-	1
Dec	94	88	2	12	12	-	14	14	3	2	1	-
Jan	137	129	9	20	29	-	10	9	4	-	1	1
Feb	65	61	5	7	23	1	5	5	1	1	1	-
Mar	106	93	4	12	39	-	9	9	1	-	-	2
Total	1104	1038	68	100	239	9	180	174	12	24	4	16

Salmonella Species Isolated from Patients with Acute Diarrhoea in Thailand
3 April 1972 - 30 March 1973

Species	Thai National			American National		
	Children	Adult	Unknown	Children	Adult	Unknown
paratyphi B	3	-	-	-	1	-
derby	14	1	-	-	5	-
san diego	1	-	-	-	-	-
typhimurium	1	-	-	-	-	-
stanley	1	1	-	-	-	-
saint paul	1	-	-	-	1	-
bovis morbillicans	2	1	-	-	-	-
tananarive	2	-	-	-	2	-
newport	1	-	-	-	-	-
montevideo	-	1	-	-	-	-
mikowasina	-	1	-	-	-	-

TABLE 2 (Cont.)

Species	Thai National				American National			
	Children	Adult	Unknown	Total	Children	Adult	Unknown	Total
virchow	-	-	-	-	-	1	-	1
typhi	6	3	-	9	-	-	-	-
moscow	-	1	-	1	-	-	-	-
jamaica	1	-	-	1	-	-	-	-
weltevreden	10	-	-	10	-	-	-	-
anatum	-	2	-	2	-	2	-	2
meleagridis	3	-	-	3	-	-	-	-
Salm. Gr. B spp.	1	-	-	1	-	-	-	-
Salm. Gr. C2 spp.	3	-	-	3	-	-	-	-
Salm. Gr. D spp.	3	-	-	3	-	-	-	-
Salm. Gr. E spp.	2	-	-	2	-	-	-	-
Salmonella spp. (not identified)	2	-	-	2	-	-	-	-
Total	53	11	-	64	-	11	-	11

Shigella Species Isolated from Patients with Acute Diarrhea in Thailand
3 April 1972 - 28 February 1973

Species	Thai National				American National			
	Children	Adult	Unknown	Total	Children	Adult	Unknown	Total
dysenteriae 4	-	1	-	1	-	-	-	-
dysenteriae 5	1	-	-	1	-	-	-	-
flexneri 1	1	-	-	1	-	-	-	-
flexneri 2	15	1	-	16	-	2	-	2
flexneri 3	30	3	-	33	-	1	1	2
flexneri 4	2	1	-	3	-	-	-	-
boydii 2	4	1	-	5	-	1	-	1
boydii 4	1	-	-	1	-	-	-	-
sonnei form I	32	-	-	32	-	18	-	18
sonnei form II	3	1	-	4	-	1	-	1

TABLE 3 (Cont.)

	Thai National				American National			
	Children	Adult	Unknown	Total	Children	Adult	Unknown	Total
Alkaescens - Dispar 01	-	1	-	1	-	-	-	-
Shigella Gr. A spp.	1	-	-	1	-	-	-	-
Shigella Gr. B spp.	1	-	-	1	-	-	-	-
Total	79	9	-	88	-	23	1	24

TABLE 4
Pathogenic *Escherichia coli* Isolated from Patients with Acute Diarrhea
in Thailand
3 April 1972 - 28 February 1973

	Thai National				American National			
	Children	Adult	Unknown	Total	Children	Adult	Unknown	Total
055:B5	8	1	-	9	-	-	-	-
026:B6	13	-	-	13	-	-	-	-
0111:B4	6	1	-	7	-	-	-	-
0127:B8	8	1	-	9	-	-	-	-
0112:B11	5	-	-	5	-	-	-	-
0119:B14	49	-	-	49	-	2	-	2
086:B7	3	-	-	3	-	-	-	-
025:B19:B23	14	-	-	14	-	-	-	-
0125:B15	13	-	-	13	-	-	-	-
0126:B16	5	-	-	5	-	-	-	-
334a	25	-	-	25	-	2	-	2

TABLE 4 (Cont.)

	Thai National				American National			
	Children	Adult	Unknown	Total	Children	Adult	Unknown	Total
B 2 C	81	1	-	82	-	-	-	-
B 7 A	5	-	-	5	-	-	-	-
Total	201	4	-	205	-	4	-	4

TABLE 5
Vibrio parahaemolyticus Isolated from Patients with Acute Diarrhea
in Thailand from 3 April 1972 - 28 February 1973

Species	Thai National			American National		
	Children	Adult	Unknown	Children	Adult	Unknown
<i>V. parahaemolyticus</i> K4	-	1	-	-	-	-
<i>V. parahaemolyticus</i> K9	-	1	-	-	-	-
<i>V. parahaemolyticus</i> K11	-	-	-	-	2	-
<i>V. parahaemolyticus</i> K12	-	-	-	-	1	-
<i>V. parahaemolyticus</i> K13	-	-	-	-	1	-
<i>V. parahaemolyticus</i> K15	-	1	-	-	-	-
<i>V. parahaemolyticus</i> K25	-	-	-	-	1	-
<i>V. parahaemolyticus</i> K39	-	-	-	-	1	-
<i>V. parahaemolyticus</i> K56	-	1	-	-	-	-
<i>V. parahaemolyticus</i> Untyped	-	5	-	-	10	-
Total	-	9	-	-	16	-

6. An Outbreak of Gastrointestinal Disease at the RTAFB Udorn, Thailand.

A three day epidemiological consultation took place in November, 1972 at the RTAF Base at Udorn. This visit was prompted by the admission to the 432nd USAF Hospital of six acutely ill patients, USAF personnel, with symptoms of chills, fever, abdominal pain, and diarrhea.

An initial survey showed an increase in both the number of outpatient visits and in the number of hospital admissions for gastrointestinal problems with the above symptoms. Consequently attack rates were calculated for the various units on post, and the hospital admission rate by unit was determined. The unit with the highest admission rate and second highest attack rate was subjected to a chunk sampling. Although several members of the unit complained of malaise and diarrhea, the carrier rate for enteric pathogenic organisms based on rectal swabs was zero.

In four of the hospitalized patients, Shigella sonnei organisms were cultured from stool specimens. Extensive questioning of these four individuals failed to reveal any common food or water source history. While a fecal-oral mode of transmission was hypothesized no particular defect in sanitary practice was found in spite of extensive examination of food handlers, mess halls, and water points.

7. Transition of A Kanagawa Negative Isolate of Vibrio parahaemolyticus to Kanagawa Positive.

The ability of strains of V. parahaemolyticus to haemolyze human or rabbit red blood cells in Wagutsuma agar has been used to signify pathogenicity for humans. (2) (3) This test is based on the fact that some investigators have noted that isolates of V. parahaemolyticus obtained from human diarrhea sources are rather universally haemolytic (Kanagawa positive) while those from natural sources (sea foods, sea water) are usually nonhaemolytic. Our findings in Thailand, however, show that the vast majority of natural isolates from the warmer coastal water of the Gulf of Siam are predominately Kanagawa positive - as are our isolates from human diarrhea. (4)

While there is no data indicating that the route of human

infection is other than the eating of contaminated sea foods; the question that remains is how does apparently nonpathogenic (Kanagawa negative) V. parahaemolyticus become pathogenic (Kanagawa positive)?

In order to study the possible development of a beta haemolysin in a Kanagawa negative strain of Vibrio parahaemolyticus, this laboratory obtained a Kanagawa negative isolate (F/674) in December 1972 from Dr. Richard J. Gilbert of the Central Public Health Laboratory in England. This isolate was originally from a natural source in Japan. Having in our laboratory, for the first time, a 100% Kanagawa negative strain of V. parahaemolyticus, we designed an experiment to study any possible natural transition from Kanagawa negative to Kanagawa positive in this isolate.

V. parahaemolyticus, strain F674, was grown in brain heart infusion (BHI) broth with 3% NaCl to a density of 10^7 cells/ml. A series of mouse passages was initiated. For each mouse passage, 0.5 ml of this culture was injected into the peritoneal cavity of each of two 21-33 gram mice. Within 4-5 hours, the animals were clinically ill and often had bloody stools. We were, however, never able to recover V. parahaemolyticus from these stools. Within 7-8 hours after the intraperitoneal injection, the mice were usually dead. Dead mice were stored at 4°C overnight and aseptically opened for examination the next morning, 24 hours after the initial inoculation.

Opened mice were examined and cultures made from the peritoneal cavity onto thiosulfate-citrate-bile salts (TCBS) agar. Pure cultures of V. parahaemolyticus, F/674, were always obtained from these animals. In the instance of obviously severe trauma, possibly resulting in peritoneal contamination from the intestine, the specimen was withheld from the study. A second cotton swab was then used to absorb additional material from the peritoneum. This swab was placed in 2 ml of BHI with 3% NaCl and swirled about in an effort to wash the material from the swab. The V. parahaemolyticus from this material was considered as a complete mouse passage. 0.5 ml of this material, was again injected into the peritoneal cavity of mice for another mouse passage. The entire process was repeated. The same culture was also immediately tested for haemolytic activity on Wagutsuma agar.

Table 1 shows the observation of haemolysis on Wagutsuma agar from duplicate mouse passages. Controls included inoculation onto the Wagutsuma agar of a nonhaemolytic Escherichia coli, and of a haemolytic isolate of V. parahaemolyticus. Each plate was also inoculated with the F/674 strain that had not been passed in the mouse. While the test occasionally appears somewhat insensitive, in every case this isolate of unpassed F/674 was either nonhaemolytic or "±". In no case was this artificially cultured strain (cultivated on thiosulfate-citrate-bile salts (TCBS) agar, brain heart infusion with 3% NaCl, etc.) more haemolytic than the identical strain maintained in vivo in the mouse.

Table 1.

Haemolysis of Vibrio parahaemolyticus
F/674 after passage in mouse peritoneum.

Mouse Passage	F/674		Control	
	Mouse A	Mouse B	1*	2*
0	-	-	-	3+
5	±	±	-	3+
8	±	1+	-	3+
10	1+	±	±	3+
13	2+	4+	-	4+
17	2+	2+	±	3+
21	2+	3+	-	3+
24	3+	3+	±	4+
28	2+	2+	-	3+
30	2+	2+	-	2+
34	3+	-	-	2+
41	2+	2+	±	4+

1* V. parahaemolyticus F/674 maintained on artificial media.

2* V. parahaemolyticus, Kanagawa positive, Thai source.

It has consistently been our experience that variations in the osmotic fragility of the rabbit or human erythrocyte affect the outcome of the haemolysis test. Results of the Kanagawa test in our laboratory are always more varied (usually more sensitive) after the Wagutsuma agar is more than eight days old. In order to alleviate the possibility that our media contributed to the above results, isolates of Vibrio parahaemolyticus were sent to other laboratories in the United States. These facilities confirmed our results on unidentified strains of our test organisms. We also feel confident that our media, manufactured each week in our laboratory, gives more consistent results than the commercially available media.

Additional work is in progress to define the haemolysin in these in vivo passed strains. The work presented here is a preliminary study and only a beginning step in the determination of why human isolates are Kanagawa positive and natural isolates (presumably the source of human infections), Kanagawa negative.

8. The Occurrence and Transmission of Vibrio parahaemolyticus in a Thai Fishing Village Population.

Gastroenteritis has long been a threat to military operations. Numerous studies to determine the agents responsible for gastrointestinal disease among United States Servicemen in Southeast Asia have been reported. In these studies there were large numbers of cases from whom a causative agent could not be isolated. Recent work indicates that the organism, V. parahaemolyticus may be responsible for a number of these "undiagnosed" cases.

We wish to define the variables important in the transmission of the organism Vibrio parahaemolyticus and to further define the disease it causes. The study population will consist of persons residing in an isolated Thai fishing village. An isolated village will increase the likelihood that sea food (the presumed vehicle of transmission) is obtained in that village market place, and will allow periodic sampling of that food. The village will be mapped and censused, at which time basic demographic information will be obtained.

A study population will be randomly selected. Families, rather than individuals, will comprise that sampling frame. Individuals in the study population will be interviewed regarding

past medical and social history. Rectal swabs will be performed on a regular weekly basis, with culture plates streaked at the house. Each week a history of gastrointestinal symptoms from the previous week will be obtained. Where indicated a food history will be taken and suspected food items will be cultured.

An initial survey of sea water, beach sand, sea food in the market place and cooking utensils, for V. parahaemolyticus, will provide data on the prevalence of the organism in natural (non-human) sources in the village area. Particular attention will be devoted to areas potentially contaminated through village waste disposal practices.

Feasibility visits were made to Ban Koh Lan during February and March 1973. There is a class 2 health center in the village staffed with a public health worker (2 years training). The first class health center on the mainland supplies a physician and a mobile health team on an irregular basis. During the initial visit contact was made with these people and their cooperation sought and readily obtained.

On the second visit in March a chunk sample of persons both ill and well was done. A total of 62 persons were questioned regarding dietary habits and gastrointestinal history. Responses to questions concerning gastrointestinal history indicate these persons average 3.6 episodes of diarrhea a year. In a random sample of the island using approximately one-quarter of the population we could expect roughly 15 diarrhea cases/week. These persons were also sampled by rectal swab for V. parahaemolyticus. (See Table 1). There were two people (3%) with positive cultures for V. parahaemolyticus. One of these was a 65 year old female, apparently asymptomatic. The other, a 2 year old child, had watery diarrhea at the time the swab was done.

Seventy percent of these persons obtain their seafood at the village market; 15% get their sea food from the mainland. Sea food is also obtained from boats (15%) and from self-catching (27%). The persons eat sea food on the average of 6.1 days/week.

Plans are underway to do a larger prevalence survey and if indicated proceed with a prospective longitudinal study.

Table 1

Ages and Sexes of Persons Submitting
Rectal Swabs.

Age	Male	Female
0-5	11	10
6-10	4	2
11-20	1	6
21-30	0	7
31+	5	16
Total	21	41

9. The Determination of Complement Levels in Typhoid Fever

Information concerning alterations in the concentrations of complement components in infectious diseases is limited. Over the past several years this laboratory has demonstrated the involvement of complement in dengue shock syndrome, a viral illness which develops in situations of antibody excess with the formation of immune complexes. Complement activation is triggered by two known mechanisms. The classical pathway involving complement components C_1 , C_4 , and C_2 is activated by immune complexes, and the recently described alternate pathway involving C_3 PA is triggered by endotoxin and immunocomplexes. Typhoid fever is caused by a gram negative bacillus, Salmonella typhosa. In cases of typhoid high levels of antibody are often reached in the continued presence of bacilli in the blood. Also S. typhosa is a gram negative organism and produces endotoxin. In typhoid fever, conditions for complement activation by both pathways are present. This study was designed to determine if a complement mediated immune mechanism is involved in the causation of typhoid fever and, if so, to determine the possible mechanisms of this activity.

Patients: All patients admitted to Bangkok Children's Hospital with a clinical diagnosis of fever of unknown origin or enteric fever, whose duration of illness was longer than one week but not longer

than 10 days were admitted to the study.

Specimens: Each patient admitted to the study had blood taken for complete blood count, Widal serology, and determination of complement components on the day of admission and on the first, second, fourth, seventh, tenth and fourteenth day of hospitalization. Also, blood for bacterial culture was obtained on the day of admission and on the 1st, 2nd, and 4th day of hospitalization. Stools were collected for culture on the same days as the blood samples were drawn. Antibiotic therapy on patients diagnosed as having typhoid fever was commenced on the 3rd hospital day.

Bacteriological cultures: All isolations were performed in the clinical laboratory of the Bangkok Children's Hospital.

Serology: The Widal test was performed by the Bacteriology department of this laboratory simultaneously on all specimens obtained from a single subject. A rise in the titer of both "O" and "H" antigens or high fixed titers ($>1:160$) of both were considered positive. Rises in either the "H" or "O" titers were considered equivocal and titers $<1:160$ with both antibodies were considered negative.

Complement Studies: Primary screening of complement level was done in the virology department of this laboratory using commercially prepared Radial Immunodiffusion tests for B_{1c}/B_{1a} . Aliquots of sera collected each day were frozen at -70°C immediately upon separation of sera for determination of other complement components.

At the time of writing, sampling and initial testing have been completed on 21 patients aged 17 months to 13 years. Of these 21 patients, 15 had serological evidence of typhoid fever by the Widal test, one was equivocal, and five had negative antibody titers. Of the fifteen positive, eleven had high fixed titers. Five of these eleven had clear reductions in B_{1c}/B_{1a} levels on admission and/or on the 1st day of hospitalization. In all of these an increase in concentration was noted starting on the 4th to 7th day and approached the mean of the 5 typhoid negative febrile controls by the fourteenth day. There was no particular serological pattern associated with the reduction and rise in B_{1c}/B_{1a} level. A search for clinical correlation with the complement findings will be undertaken.

10. Investigation of Suspected Meningococcal Meningitis at RVNAF Recruiting, Induction, and Training Centers:

Outbreaks of meningitis had occurred at twelve recruit training centers of the Republic of Vietnam Armed Forces from March through September 1972. During this period 1371 cases of suspected meningitis, including 338 deaths (case fatality ratio = 24.6%) were reported. Neisseria meningitidis was reportedly isolated from 28 of these cases. All recruits routinely received sulfadiazine prophylaxis (2 grams per day for 3 days) at the Induction Center and again at the Reception Center. Group-specific polysaccharide vaccine had not been employed.

The Office of the Command Surgeon, Headquarters, Military Assistance Command, Vietnam, requested the assistance of SMRL to verify that the disease reported as "suspected meningococcal meningitis" was caused by infection with N. meningitidis; to isolate and characterize strains of N. meningitidis causing disease and circulating in the population in which disease was prevalent; to study the variables contributing to the outbreak and severity of disease, and to make recommendations appropriate to the further study and control of these outbreaks.

Centers at which cases were still a frequent occurrence and medical facilities serving these Centers were visited. These included, in the Third Military Region, the Quang Trung Training Center (44 cases and 7 deaths in September 1972). Cong Hoa Hospital, and the Third Induction Center, adjacent to Quang Trung. In the Fourth Military Region, The Fourth Induction Center, Can Tho (24 cases, 6 deaths), Chi Lang Training Center (49 cases, 3 deaths) and Phan Thanh Gian Hospital were visited.

At each Center, following a briefing, the dispensaries, barracks complexes, and training areas were seen. Many of the physicians and training cadre were questioned. Units in which we wanted to determine the prevalence of N. meningitidis carriers were placed at our disposal. The procedures employed involved swabbing the posterior nasopharynx with bent wire Calcium Alginate swabs, streaking the swab contents on chocolate agar, placing the streaked plates immediately into CO₂ cans, and incubating at 35-37°C, as soon as possible after the cultures were taken.

At the dispensaries and hospitals, recruits admitted and

treated for suspected meningococcal meningitis were examined. Where spinal fluid or petechial isolates were available, the organism was subcultured for later studies.

Overcrowding of the barracks and mess hall facilities was observed at all Centers. Unit integrity was maintained during training sessions, but opportunities to interact with recruits from other units occurred in and around the mess hall, during the night-time political indoctrination sessions, and during sick call. The number of cadre with each recruit company varied considerably. Cadre at all Centers appeared to be well sensitized to the problem of meningitis. Screening of recruits reporting sick was performed by battalion aid-men. All recruits with febrile upper respiratory conditions were reportedly hospitalized. Therapy routinely consisted of penicillin, 20 million units per day IV drip, occasionally with chloramphenicol, 4 grams per day, added. Shock was treated with volume replacement, Solucortef, Isuprel, and bicarbonate. The majority of hospitalized recruits reportedly recovered rapidly on this regimen. Those who recovered appeared to do so completely.

Spinal fluid isolates from five patients whose disease coincided with our visit were subcultured. Patients CSP 1-3 are from Phan Thann Gian Hospital; CSP 4-5 are from Cong Hoa Hospital. A sample of healthy recruits in the Induction and Reception Centers was swabbed. Neisseria meningitidis was isolated from 115 of 209 specimens obtained (Table 1). These and the five case isolates were sero-grouped (Table 2) and sulfadiazine sensitivities determined (Table 3). These data indicate that carrier rates were high, that sero-groups "B" and "C" were most common and similar in prevalence, and that most strains were sulfadiazine sensitive. Four of the five case isolates were sero-group "B" and all were sulfadiazine sensitive.

The following recommendations were made as a result of these investigations:

1. That bacteriologic capability to confirm a greater proportion of suspected cases from Induction and Training Centers and to support a system for surveillance of new outbreaks be established.

2. That the provision of adequate sleeping space, well ventilated billets, adequate nutrition, adequate periods of sleep,

alternating head and foot sleeping arrangements, avoidance of overfatigue, and the importance of early diagnosis and treatment receive increasing command interest.

3. That the training schedule provide for adequate numbers of cadre, for the maintenance of unit integrity, and for adequate numbers of daily formations at which ill recruits could be identified.

4. That sick call and hospital admission policies remain liberal and all febrile URI patients should continue to be hospitalized.

5. That the apparent ineffectiveness of sulfadiazine may be due to sulfadiazine whose activity has decayed or to imperfect discipline. Both these possibilities needed to be studied further.

6. That utilization of Group C meningococcal vaccine was not indicated at the time, since the case strains isolated were predominately Group B.

TABLE 1
Recovery of *Neisseria meningitidis* from N/P Swabs
From ARVN Recruits (25 September- 5 October 1972)

Specimen Source	Total Specimens	Positive <u>N. meningitidis</u>
Quang Trung, 3 days following Sulfadiazine prophylaxis	29	18 (62.06%)
Quang Trung, Completing first week of training	30	21 (70.00%)
Quang Trung, Received the previous day from Huang Hai Province Recruiting Station	30	2 (06.66%)
Fourth Induction Center	30	26 (86.66%)
Fourth Induction Center, Held for physical re-evaluation	30	11 (36.66%)
Chi Lang, Completing third day of Sulfadiazine prophylaxis	30	15 (50.00%)
Chi Lang, Completing first week of training	30	22 (73.33%)

TABLE 2

Serotypes of Neisseria meningitidis Isolated
From ARVN Recruits (25 September - 5 October 1972)

Specimen Source	Serotypes (WRAIR Antisera)			
	A	B	Bo	C
Quang Trung, 3 days following Sulfadiazine prophylaxis	1	7	1	9
Quang Trung, Completing first week of training	0	4	5	12
Quang Trung, Received the previous day from Huang Hai Province Recruiting Station	0	2	0	0
Fourth Induction Center	0	16	1	9
Fourth Induction Center, Held for physical re-evaluation	0	8	0	3
Chi Lang, Completing third day of Sulfadiazine prophylaxis	0	8	1	6
Chi Lang, Completing first week of training	0	7	1	14
Case Isolates (4) Quang Trung, occurring at different times	0	3	0	1
Case Isolate (1) Can Tho	0	1	0	0

TABLE 3

Sulfadiazine Sensitivity Profiles of 120 Isolates of *Neisseria meningitidis*
Recovered from ARVN Recruits and Patients

Specimen Source	Sulfadiazine Sensitivity		
	Sensitive	Reduced Sensitivity	Resistant
Quang Trung, 3 days following Sulfadiazine prophylaxis	14	3	1*
Quang Trung, Completing first week of training	20	1	0
Quang Trung, Received the previous day from Huang Hai Province Recruiting Station	2	0	0
Fourth Induction Center	26	0	0
Fourth Induction Center, Held for physical re-evaluation	11	0	0
Chi Lang, Completing third day of sulfadiazine prophylaxis	15	1	0
Chi Lang, Completing first week of training	17	5	0
Case Isolates (5)	5	0	0

* Tube dilution revealed MIC for this isolate to be 400 mcg/ml.

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Task 00, Tropical Medicine

Work Unit 002 Tropical and subtropical military medical research

Literature Cited.

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Project 3A062110A831 TROPICAL MEDICINE

Task 00, Tropical Medicine

Work Unit 002 Tropical and subtropical military medical research

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1. Adenosine Triphosphate Level of In Vitro Erythrocyte-Free P. falciparum.

These studies were undertaken to determine the ATP levels of erythrocyte-free P. falciparum during the course of maturation in vitro.

Extensive studies are underway by many investigators to explore the ATP Malaria Hypothesis¹⁻⁴. Many investigators have theorized that erythrocytic ATP levels of the host may play an important role in supporting the initial growth of the parasite, and that erythrocytes with low levels of ATP would be less viable and tend to rupture easier than erythrocytes with high ATP levels. In addition, a positive direct correlation of the erythrocytic ATP level with the severity of the primary clinical course of malaria is evident. Experiments in humans have been limited to the very early stage of infection to avoid undue risk to volunteers. Although supporting data may be obtained from experiments on Rhesus monkeys, the effects of low ATP levels on inhibition of parasite development have not been elucidated. It has been suggested that in addition to the fact that red cells containing low ATP level may not survive to support the parasites, the initial ATP requirements of the parasite may be more than the cell can provide.

With the technique of in vitro culture of native P. falciparum developed here at this laboratory, the growth of parasites can be followed through maturity. The cell-free parasitic ATP level will be determined at different stages of development throughout the culture period and related to the morphology. The increase in parasitic ATP levels during the course of maturation in vitro will indicate the direct relationship between the intraerythrocytic parasites and their host cells.

The in vitro culture system of P. falciparum is the same as that previously described⁵ with the exception that ¹⁴C-isoleucine is not added to the culture media. The contents of several wells will be lysed by one of three methods being considered, under controlled conditions to prevent lysis of the parasites. Ammonium chloride lysis, selective pressure and immune lysis are three techniques to be attempted. The ATP levels of the freed parasites will be determined by the luciferase enzyme method as described by Stanley⁶.

The luciferase-ATP measurement system is being standardized and selective lysis studies are underway. No conclusive data have been produced to date.

2. A Quantitative Study on the Level of Adenosine Triphosphate in Human Erythrocytes by Luciferase Enzyme.

The objective of these studies was to establish a quantitative assay of ATP in human erythrocytes for determination of normal erythrocytic ATP levels in a Thai population and their relationship to malaria infections.

It is known that there is considerable variation in the levels of erythrocytic ATP between individuals in a population, and that this level is constant in healthy individuals^{1,3}. Comparative studies in American Negroes and Caucasians indicated the existence of different mean quantities of erythrocytic ATP between these two groups⁷. Since the gene pool of the American Negroes is derived from an African Negro stock exposed to malaria for many generations, the lower mean levels of ATP in this group suggest selection pressure for malaria. Further studies revealed that there is a strong positive correlation between the erythrocytic level of ATP and P. falciparum parasitemia⁷. In human as well as simian infections, high ATP levels were directly associated with relatively high peak parasite counts². It has been suggested that the protective mechanism against malaria infection may result from the following:

a. Erythrocytic ATP levels of the host play an important role in supporting the initial increase of parasitemia. With the lower level of ATP, a retardation of the primary increase in parasitemia will be seen, resulting in a less severe clinical course of infection.

b. The role of ATP in maintaining metabolism and viability of living cells indicates that red cells with low ATP levels would be less capable of maintaining their viability. This will, in turn, result in inability of the intraerythrocytic asexual parasites to develop through completion and rupturing of the parasitized erythrocytes may occur prematurely.

It is our aim to investigate erythrocytic ATP levels in Thai populations continuously exposed to malaria infection and to compare them with populations from non-endemic areas. Results obtained may reveal a significant protective mechanism against P. falciparum infection.

A technique for quantitative assay of erythrocytic ATP utilizing a firefly luminescence method described by Stanley and Williams⁶ is being established. A standard curve of ATP is obtained by adding an aliquot of fresh extract of desiccated firefly lanterns to various known concentrations of ATP in phosphate buffer, pH 7.4. The resulting light pulses are counted in the liquid scintillation spectrometer.

A comparative study utilizing fresh and frozen (in liquid nitrogen) blood specimens is in progress. Although the firefly luminescence method assay of ATP has advantages over other techniques described, a limitation of this procedure is that blood specimens must be processed soon after collection to prevent a reduction of ATP levels. Modifications of the method of red cell lysate preparation are being developed. It is anticipated that results obtained in these experiments will provide a means for measurement of ATP in erythrocytes collected from field surveys.

3. Evaluation of Refinement of a Radioimmunoassay for the Quantitative Estimation of Malarial Antibody.

Experiments are in progress to refine, field test and evaluate a radioimmunoassay for quantitative estimation of specific malarial antibody. A radioimmunoassay was developed at WRAIR for the purpose of quantitatively estimating specific malarial antibodies in human serum. Specific antibody is measured by inhibition of binding between monospecific antiglobulin and radioiodinated purified immunoglobulin. Preliminary experiments indicated that the method can be used to detect quantities of specific antibody as low as 0.05 $\mu\text{g/ml}$ and purified IgG in concentrations of 0.01 $\mu\text{g/ml}$. The development of the assay and tests for sensitivity and reliability were accomplished using pools of sera obtained from individuals living in areas of high endemicity. The assay proved to be reliable and the data easily reproducible.

The insolubilization method chosen for this assay was that of sensitizing sheep red blood cells (SRBC) with the antigen and using a prescribed number of cells per tube as the standardized antigen. Three major problems are inherent in this system: (1) the actual

amount of antigen bound to the cells is difficult to determine; (2) different antigen preparations are difficult to standardize; and (3) all sera have to be adsorbed onto SRBC prior to being used in the assay. Due to these problems, a new means of insolubilizing the antigen was sought. Catt and Trager⁸ found that immunoglobulins would bind to polymers of plastics which are used in making disposable culture tubes. Further experimentation indicated that other proteins would also bind. The malaria antigen used in the radioimmunoassay is basically protein in nature, therefore this technique was selected for evaluation as a better means of antigen insolubilization.

The antigen preparation is radiolabeled with ¹²⁵I and varying concentrations are used to coat 12 x 75 mm polypropylene tubes. The tubes are positioned so that a prescribed amount of fluid will cover the same surface area of the tube each time. Care is taken not to splash any antigen on the side of the tube. A coating period of 2 hours at room temperature has been shown to be the most satisfactory of those tried. No significant increase in antigen binding is observed with longer periods of time. A concentration of 10 µg/ml was found to provide optimal coating. Approximately 11% of the radiolabeled antigen is bound to the tube at this concentration. After the antigen solution is removed, the tube is washed 5 times and filled with a 3% human serum albumin-phosphate buffered saline solution to fill the remainder of the binding sites and to remove the loosely bound antigen.

Repeated experiments with this tube coating process have shown that when 0.5 ml of a 10 µg/ml coating solution is used, 0.285 µg (±0.023) is bound to the tube. A pH of 7.5 is optimal as opposed to that found by Catt and Trager (pH 9.5 - 10.0). Experiments conducted at 40°, 22° and 37°C indicate that 22°C (room temperature) is optimal for binding. Coated tubes have been stored at 40°C for as long as 7 days and no loss of antigenicity was observed. Experiments are presently being conducted to determine how long these coated tubes can be stored at 40° and -70°C without loss of antigenicity.

The protocol for the radioimmunoassay requires a standard curve to be established for each assay conducted. In order to have comparable conditions existing in both the standard and the antigen tubes, a series of experiments were conducted on the binding capability of purified human IgG. These experiments indicated that the same optimal conditions existed for the IgG as for the malaria antigen. It is possible to bind approximately 50% of varying concentrations (0.25 to 4.0 µg/ml) of ¹²⁵I-IgG to the tubes and the repeatability is within a 5% error.

Preliminary experiments with the pooled immune serum indicate that results are comparable to those obtained with the SRBC carrier. Initial experiments with individual serum samples collected in Thailand indicate that the tube-bound malaria antigen will detect specific

antibody in relatively low concentrations (5 µg/ml). Future experiments will be designed to increase the sensitivity.

4. In vitro Drug Sensitivity Testing of Plasmodium falciparum.

In the previous Annual Report⁹, the in vitro system for drug sensitivity testing of human P. falciparum utilizing radioactive isoleucine as a growth marker was described. The results indicated a low culture failure and a good reproducibility. Comparison of parasite C¹⁴-isoleucine incorporation and the morphology of the parasite during the course of maturity indicated an excellent correlation. The present investigation was done to obtain additional data on chloroquine sensitive P. falciparum for establishment of a base line growth curve for a susceptible strain.

It has been previously reported that strains of P. falciparum resistant to chloroquine are widely spread in Thailand. In some areas nearly 100% of the P. falciparum is resistant⁹. The parasite source for cultures is primarily from Choburi Province, where the prevalence of resistant P. falciparum also approaches 100%. Results obtained from in vitro cultures support this observation, although the in vivo comparison has not been possible in every case.

In the first part of this study a test for assessing the in vitro susceptibility of P. falciparum has been described. The culture system utilizes the protein incorporation of C¹⁴-isoleucine as a growth marker. Results obtained indicate a good reproducibility with the multiplication rate of 2:1 or better and culture failure was low. Patients admitted to Yala Provincial Hospital were used for in vivo - in vitro comparison studies, but the number of patients for a clinical follow-up was not sufficient to make an adequate number of observations on susceptible parasites.

Collaborative studies were established with the Faculty of Tropical Medicine, Mahidol University, Bangkok. Blood specimens obtained from patients with native P. falciparum were processed in in vitro culture utilizing the previously described technique. An aliquot of each specimen was washed and preserved in liquid nitrogen for later reference when required. A standard therapeutic course of chloroquine was administered to the patient and parasitemia was followed for evaluation of clinical response. The type of chloroquine resistance was established, utilizing WHO¹⁰ criteria for definition of resistance. Clinical response was later compared with the in vitro growth curve determined from C¹⁴-isoleucine uptake. The in vitro drug sensitivity test was performed on 35 blood specimens, obtained from Somdej Sri Racha Hospital and the Faculty of Tropical Medicine, Bangkok. The growth curves obtained from the C¹⁴-isoleucine parasite incorporation were available from 25 in vitro cultures of which 20 were from Somdej Sri Racha Hospital and 5 from the Faculty of Tropical Medicine. Among

these, 24 in vitro cultures were resistant to chloroquine and 1 was susceptible. Comparative results for in vivo and in vitro studies were obtained from 5 cases admitted to the Faculty of Tropical Medicine. The resistance of P. falciparum to chloroquine was observed both in vitro and in vivo in 4 cases. The patients were treated with other antimalarials after the assessment of chloroquine resistance. One case assessed as chloroquine susceptible in vitro is still under clinical observation. Parasitemia in this case lasted 24 hours after commencement of a standard therapeutic course of chloroquine.

It is obvious that the in vitro studies described have the limitation of depending on a natural human infection for the supply of infected blood. With the high prevalence of resistant infection, a base line growth curve of susceptible parasites will support the evaluation process of the in vitro test. Attempts are being made to obtain a sufficient number of samples of susceptible parasites to provide a reference for comparison with the quinine growth curve.

Additional studies are being done with the in vitro sensitivity test utilizing the currently reported effective antimalarials for comparison with quinine. The plasma levels for each antimalarial are being used as reference for the concentration to be tested in vitro. Antimalarials presently being tested are:

1. Pyrimethamine
2. Dapsone
3. Sulfadoxine
4. Sulfadoxine in combination with pyrimethamine
5. Primaquine phosphate

The morphological variations of the parasites observed during the incubation period in the presence of various antimalarials will be correlated with the growth curve of C^{14} -isoleucine incorporation. This observation, if possible, will be made on susceptible parasites and be correlated with the in vivo response.

5. In vitro Infection Rate of Plasmodium falciparum in Human Erythrocytes with Different Hemoglobin Types.

It has been proposed that resistance to P. falciparum infection is related to different natural factors. Among these, the red blood cell defects, namely Hb S, Hb E, thalassemia and G-6-PD deficiency have been extensively studied.¹¹⁻¹⁷ Evidence supporting the theory that Hb S deficiency conferred natural resistance to the host were convincing, but controversial results were obtained in thalassemia and Hb E.¹⁸

Significant numbers of individuals with different hemoglobin types and thalassemia exist in Thailand. A number of such cases are

available at the Division of Hematology, Department of Medicine, Siriraj Hospital. Extensive studies on hematological aspects of hemoglobin differences have been performed at the Division. A collaborative study has been designed to study these differences utilizing the capability of in vitro culture techniques developed at this laboratory.

Heparinized blood from patients with native infection of P. falciparum is the source for parasites. Blood group and hemoglobin type are established and hematological investigations are performed on each case. Compatible uninfected blood with the various hemoglobin types are included in in vitro culture serving as target for reinvasions. The proportion of erythrocytes containing different hemoglobin types in each culture will be determined.

The proportion of cells in each in vitro culture is prepared as follows:

	Row 1	Row 2
Patient infected cells (AA)	1 volume	1 volume
Target cells (AA)	1 volume	-
Recipient cells (AE)	-	1 volume
% of AA cells	100	50
% of AE cells	-	50

Erythrocytes containing different Hb types can be differentiated, e.g., Hb E containing erythrocytes and Hb F containing erythrocytes can be distinguished by the technique of Hb staining by differential elutions. The parasite density rate of different hemoglobin containing erythrocytes can be determined by counter-staining with Giemsa stain.

If the results obtained from such cultures reveal a lower in vitro infection rate of thalassemic erythrocytes or erythrocytes containing abnormal hemoglobin, it will suggest that intraerythrocytic conditions of these cells will not readily support the parasite. However, if an equal infection rate is found, a conclusion against this hypothesis of protection cannot be made. The selective mechanism against malaria infection may be operating outside the red blood cells or by a combination of the red blood cells and other organs, e.g., the reticuloendothelial system, especially the spleen.

To avoid such controversial results, our experiments are designed differently from the previous described in vitro systems.¹⁹ A later stage of P. falciparum trophozoites will be used in our study to allow a longer period of observation of the young merozoites after reinvasion.

Collections of infected blood are being made at the present time. A portion of each sample is cultured immediately while the remainder

is frozen in liquid nitrogen for later comparison. The initial experiments are to determine if freezing will have an effect on cells with different types of hemoglobin. The infected cells of certain hemoglobin types are difficult to obtain at some times of the year, and therefore, adequate preculture storage methods are necessary.

6. A Malaria Survey Conducted at Panom Sarakam in Cha Choeng Sao Province, Thailand.

A malaria survey has been performed on a stable population of workers and families located in the forest at a lumber camp. An earlier visit to this lumber camp indicated that this area may be satisfactory for detailed study of the immune response to malarial infections. Although the population is relatively small, it is stable and would provide a continuing source of material for several studies. The camp is located within a 2 hour drive from the laboratory thereby providing good accessibility. Migration is limited and the population is willing to participate in a malaria study.

The survey was conducted over a two day period and included a majority of the workers and their families. The name of each individual was recorded and blood samples were obtained by venipuncture on everyone possible. Finger or heel sticks were made on infants. Two thick and thin blood smears were made for each individual, hematocrit tubes were filled, and a collection of the blood was made on filter paper (Whatman No. 1) in a predescribed area. The remainder of the blood was allowed to clot and the serum was removed. After the hematocrit reading was obtained, the tubes were cut at the cell-plasma interface, both ends of the plasma containing portion of the tube sealed with clay and the tube placed on ice. Upon return to the laboratory the tubes were emptied and the plasma frozen. The filter papers were appropriately labeled, allowed to dry and placed in air tight containers. The serum from the clot, plasma from the hematocrit tubes and the filter paper extracts will be assayed by radioimmunoassay to determine antibody levels, and results of the three collection methods compared. Each individual was examined for enlargement of the spleen and a history of malaria infections was obtained. With the individuals complaining of fever, one thick blood smear was immediately stained with aqueous Romanowsky's stain and examined for the presence of parasites. All individuals with complaints were either treated or referred to one of the local hospitals for further examination and treatment.

The resident population consists of approximately 400 individuals of which 100 are classified as full time employees. An additional 100 employees live off the plantation and commute to work on a daily basis. The age at which an individual enters the jungle to work on a full-time basis is 15; however, children as young as 10 are employed on a part-time basis to clear weeds and underbrush. A total of 327 individuals were included in the survey. Venipunctures were done on 262

individuals with hematocrit tubes being filled by finger or heel sticks on 25 more, giving a total of 297 collections of serum and/or plasma and corresponding filter paper collections. A total of 315 individuals were examined for spleen size (Table 1) with a spleen rate of 10.48%.

Table 1. Results of Spleen Examinations

Category	Spleen Size					Rate
	0	1	2	3	4	
Males under 15	23	0	2	5	1	11.27%
Females under 15	57	0	1	2	0	5.00%
Males 15 and older	95	1	5	11	0	15.18%
Females 15 and older	67	1	0	4	0	6.94%
Total	282	2	8	22	1	10.48%

A total of 294 thick and thin smears were examined twice by WHO standards with 59 positive for a parasitemia rate of 20.07% (Table 2).

Table 2. Results of Blood Film Examination

Category	NPS*	<u>P. falciparum</u>	<u>P. vivax</u>	Mixed	Rate
Males under 15	44	7	3	0	18.52%
Females under 15	41	6	1	0	14.29%
Males 15 and older	84	20	10	0	26.32%
Females 15 and older	66	8	2	2	15.38%
Total	235	41	16	2	20.07%

*NPS - No parasites seen

The asexual parasitemia rate was low (Table 3). Both palpable spleens and parasitemia were observed primarily in individuals 15 years of age and older. Approximately 18% of the observed parasitemias

were in children of age 14 and under. Hematocrit values were within a normal range (35-50%) for the majority of the persons included in this study. Approximately 75% of the infections were asymptomatic as indicated by the medical histories.

Table 3. Observed Gametocytemia

Category	<u>P. falciparum</u>	<u>P. vivax</u>	Mixed
Sexual parasite rate	0.0%	0.0%	0.0%
Asexual parasite rate	1.0%	0.0%	0.0%
Mixed	12.88%	5.76%	0.36%
Total	13.88%	5.76%	0.36%

Data from the radioimmunoassay are not yet available to determine the specific antibody levels; however, the relatively high prevalence of malaria infections during the dry part of the year indicates that this area will be adequate for a study of the immune response as measured by specific antibody levels and by in vitro evaluation of the antibody molecule.

7. Evaluation of the Effectiveness of Experimental Antimalarial Drugs in Rhesus Monkeys Infected with Plasmodium cynomolgi.

These studies are designed to determine the efficacy of experimental antimalarial drugs in rhesus monkeys infected with blood-induced Plasmodium cynomolgi. The experimental drugs are furnished by the Division of Medicinal Chemistry, Walter Reed Army Institute of Research.

Rhesus monkeys (Macaca mulatta) of either sex, weighing 2-4 kg. are infected by the intravenous administration of 5×10^8 parasitized cells from a donor monkey infected with Plasmodium cynomolgi strain-B. Four days later, when the parasitemia is well-established, a 7 day course of daily drug administration is initiated. Drugs are routinely prepared in a suspension and given orally by stomach tube, but other methods of treatment are used on occasion. The effectiveness of the drug is determined by following the parasitemia over a thirty day period. At the end of thirty days, monkeys with negative blood smears are splenectomized. Only those monkeys which remain negative for 30 days after splenectomy are classified as CURED. In addition to parasite counts, each monkey is observed daily for clinical signs of

Table 1
Minimum Curative Doses of Experimental Antimalarial Drugs
in Rhesus Monkeys Infected with Plasmodium Cynomolgi

TYPE OF DRUG	WRAIR DRUG IDENTIFICATION NO.	MINIMUM CURATIVE DOSE (mg/kg)	TYPE OF DRUG	WRAIR DRUG IDENTIFICATION NO.	MINIMUM CURATIVE DOSE (mg/kg)
4-Aminoquinolines	WR 1544 (Chloroquine)	5.0	Pyridinemethanols	WR 148946	100.0
	WR 2975 (Primaquine)	Not curative		WR 154904	3.16
	WR 161085	100.0		WR 172435	10.0
	WR 180411	31.6		WR 175039	3.16
Quinolinemethanols	WR 30090	100.0	2,4-Diaminoquinazolin- ines	WR 178919	10.0
	WR 166391	Not curative		WR 182123	3.16
	WR 171668	31.6		WR 141871	10.0
2,8-Trifluoromethyl Quinolinemethanols	WR 142490	10.0		WR 154928	1.0
	WR 177504	10.0		WR 158122	1.0
	WR 177602	10.0		WR 159412	0.1
	WR 183544	31.6		WR 162878	0.1
	WR 183545	100.0		WR 164104	0.1
	WR 183546	31.6		WR 180153	0.316
	WR 183606	31.6		WR 181953	1.0
	WR 184806	10.0		WR 448 (Dapsone)	10.0
Phenanthrenemethanols	WR 122455	7.0	Miscellaneous	WR 25187 (Prodigiosin)	Ineffective orally
	WR 143803	100.0		WR 49808 (Menoctone)	Ineffective orally
	WR 146459	31.6		WR 150008	31.6
	WR 150726	31.6		WR 178340	Ineffective
	WR 165355	10.0		WR 178448	10.0
	WR 165533	31.6		WR 179305	31.6
	WR 171669	3.16			
	WR 175412	3.16			
	WR 178979	10.0			
	WR 185020	31.6			
	WR 190420	3.16			

drug toxicity or intercurrent disease. Necropsy examinations are performed terminally.

Each experimental drug is evaluated over a series of doses to determine a minimum curative dose, a minimum effective dose and a maximum tolerated dose. Normally two monkeys are treated at each dose level, with doses spaced 0.5 Log 10 apart (316, 100, 31.6, 10.0, 3.16, 1.0 mg/kg, etc.).

During the 15 month period terminating 1 April 1973, 47 drugs were evaluated for antimalarial efficacy in P. cynomolgi-infected rhesus monkeys. A tabulation of the drugs studied and their minimum curative doses is presented in Table 1.

8. Immunochemical Studies of Gibbon Anti-DNP Antibody.

The affinity of antibody produced in gibbons against the hapten dinitrophenol (DNP) is being studied. The gibbon has been used as an experimental animal for studies involving the immune response to various pathogenic organisms. Little is known about the kinetics of a specific antibody response. To learn more about antibody produced in the gibbon a series of experiments were designed using a hapten system for purposes of simplicity. Dinitrophenol (DNP) was selected as the hapten since this system has been defined in several other species. A test antigen of DNP- protein was prepared by linking DNP to bovine gamma globulin (BGG) by nucleophilic substitution using a sulphonic acid derivative, sodium dinitrobenzyl sulphonate. The epsilon-NH₂ groups of lysine residues are essentially the only substitutions made in this system thus allowing better definition of the hapten-carrier combination.

Each animal was injected subcutaneously with 2.5 mg DNP-BGG (mixed 1:1 with Freund's complete adjuvant). A minimum of 4 injection sites was used. A second injection of DNP-BGG in Freund's incomplete adjuvant was administered 30 days after the initial dose. A third inoculation of alum precipitated DNP-BGG was administered 60 days after the initial injection. Each animal was bled once a week throughout the inoculation schedule.

Five gibbons were selected for these experiments, and 3 have been immunized through the first two injections. Two gibbons were kept as controls and were bled according to the immunization schedule. Immuno-adsorbents of DNP-lysine conjugated to sepharose have been prepared and a new technique for elution of purified antibody has been tested and found to be better than previous techniques (Hill, 1972).²⁰ This particular technique has not been reported for a hapten-antibody system. Experiments have been designed for adsorbing anti-DNP antibody from each of the successive bleedings from each animal, and for determining the affinity of the antibody by the fluorescence quenching

technique. The antibody affinity data obtained in these experiments will be correlated with the agglutinating, precipitating, and complement fixing activity of these same sera.

9. Immunodiagnosis of Filarial Infections

Studies are in progress to determine the incidence of *Dirofilariasis* in humans and dogs with above normal numbers of circulating eosinophils.

Eosinophilia frequently appears in patients with certain parasitic infections, allergies and dermatosis. The actual role of the eosinophil leukocyte has not yet been elucidated, but there is evidence that the mechanism of eosinophilia may be mediated by immune processes. Most of the experimental evidence for this is based on experimental infections with *Trichinella spiralis* in rats and mice.²¹ The clinical syndrome, Tropical Eosinophilia, has been recognized for many years and high titers of antibody against filarial antigens have been detected in active cases. These titers subside after treatment with diethylcarbamazine.^{22,23} This syndrome has been reported from many areas, including a large part of Southeast Asia. The inciting organism is believed to be *Wucheria bancrofti* in many instances.²⁴

An increase in circulating eosinophils is often observed in Americans in Thailand without any other symptoms of Tropical Eosinophilia. Exposure to *W. bancrofti* is limited in Bangkok, but exposure to *Dirofilaria immitis*, a parasite of dogs, could be high as the incidence of heartworms in dogs in Bangkok is high. A survey of outpatients seen at the U.S. Army Hospital, Bangkok, with an above normal number of eosinophils was performed to determine if serological titers for *D. immitis* antigens could be demonstrated.

All outpatient records were screened to select patients with eosinophil counts above 8 percent. These individuals were bled and a portion of each serum sample kept while the remainder was processed by the Knott's Concentration Method for circulating microfilaria. The sera were tested against *D. immitis* antigen by the indirect hemagglutination assay (IHA) using antigen prepared from a saline extract of adult worms. Specificity of the assay was determined by using sera of patients with known helminthic infections (e.g., gnathostomiasis) in assays against the filarial antigen and by using antigens of other parasites and the sera of patients positive for filaria. All sera will be tested in the Soluble Antigen Fluorescent Antibody Test (SAFA) as an additional verification of the positives obtained with the IHA. Control sera (100) were obtained from the U.S. Army Hospital, Bangkok, from outpatients demonstrating less than 4% eosinophils. When a member of a family had eosinophilia, the remaining members of the family and pets, if any, were included in the study. Dogs which were brought to the Veterinary Clinic were routinely checked for *D. immitis* and those that were positive or demonstrated eosinophilia were included in the

Table 1: Results of IHA for Human Samples

Per cent Eosinophils	0-4	5-7	8-11	12-15	16-19	20+	Total
Observations	133	36	97	41	19	23	349
IHA Positive	29 ^a	9 ^b	6	4	4	5	57
Per cent Positive	21.8	25.0	6.2	9.8	21.7	21.7	16.3

a. 28 individuals in this group were members of family groups sampled after the index individual was found to have eosinophilia.

b. All positives in this group were members of family groups sampled after the index individual was found to have eosinophilia.

Table 2: Results of IHA for Canine Samples

Per cent Eosinophils	0-4	5-7	8-11	12-15	16-19	20+	Total
Observations	2	3	8	9	7	7	36
IHA Positive	1	3	8	8	3	7	30
Per cent Positive	50.0	100.0	100.0	88.9	42.9	100	83.3

study with the owners and families.

To date, 349 human samples and 36 canine samples have been tested in the IHA. Of these samples 57 (16.3%) human (Table 1) and 30 (83.3%) canine (Table 2) samples were serologically positive for filariasis. Of the 177 human samples with 8% eosinophilia or greater 19 were positive (10.7%).

Of the 29 IHA positive sera from individuals with less than 4 percent eosinophils, 28 were from members of family groups which had been sampled based on the eosinophilia of the index member. There were no IHA positive sera among the 100 control sera obtained from the hospital laboratory.

Future plans call for the data to be statistically analyzed to determine whether it supports the hypothesis that individuals in close contact with infected dogs will develop eosinophilia and antibody to the microfilaria. No microfilaria were isolated from human samples; however, a number of the canine samples had microfilaria. Family groups will be studied and the canine-human relationship investigated. A number of the sera obtained from military guard dogs were IHA positive and the handlers of these dogs will be included in the study. All sera presently on hand will be tested with the SAFA test and all future collections will be tested with both the IHA and the SAFA.

10. A Survey of Rodent Parasites in Southeastern Thailand.

The objective of this study was to survey a rodent population, primarily Bandicota indica, to determine what intestinal and blood parasites were present and to ascertain if B. indica could be acting as a reservoir for parasites which infect man.

Traps were set in both rural and urban areas for a period of 3 months. Material for parasitological study was processed as follows:

a. Intestinal contents: The gastrointestinal tracts of rodents trapped in the field were placed in 10% Buffered Formalin and sent immediately to SMRL. In the laboratory the intestines were removed from the fixative and excised longitudinally to expose their contents. This material was then placed in a Kimax conical graduated cylinder. Tap water was added to the one liter mark and the material allowed to settle. The supernatant was removed and the above procedure repeated until a clear supernatant could be observed. After the last wash the supernatant was removed and the sediment placed in a petri dish and examined for adult parasites.

b. Cecal contents: Cecal material was processed according to the Ritchie modification of the Formalin Ether Concentration Technique. The resulting sediment was then treated with Lugol's iodine and examined microscopically for parasites.

c. Blood smears: Thick and thin smears were made from the blood of decapitated rodents. After air drying, the smears were fixed with methanol and shipped to the main laboratory. After staining with a 2% solution of Giemsa stain (commercial), the slides were examined microscopically for blood parasites. Fifty thick microscopic fields were examined before reporting a slide negative.

In two provinces in Southeastern Thailand 704 rodents were trapped (Table 1a). Of these, 514 (73%) were Bandicota indica. The parasites found in the study are shown in Table 1b and are grouped according to province in Tables 2a, 2b and 3a, 3b.

All the nematodes, which accounted for the majority of the parasites found, are routinely reported in rodents. Some have been reported in man but only rarely. One hookworm, Cyclodontostomum sp., which has not previously been reported in Thailand, was found in 28.4% of the B. indica trapped in the Ban Thapong area. Further study is required to determine its possible relationship (if any) to man. Another organism, an acanthocephalan (Moniliformis moniliformis), has been reported in man before but is considered by some as an incidental parasite.

All three cestodes found in the study can be considered as being transmissible to man. One tapeworm (Raillietina sp.) has been discovered among infants in Thailand (Chandler and Pradatsundarasar, 1957).²⁵ This finding is not surprising since this tapeworm presumably requires arthropods such as cockroaches as an intermediate host and some of the children infected had a history of playing with cockroaches. It is also of interest to note that all nine cases of Raillietina infection found in Thailand up to 1960 have been children under 5 years of age (Pradatsundarasar, 1960).²⁶ The two remaining cestodes H. nana and H. diminuta have been reported before in man. H. diminuta, in fact, was reported in a study of the Thai population in Bangkok (Baughn and Morales, 1971).²⁷

Only one fluke of the genus Echinostoma was found in our survey. This parasite has been previously reported in rodents in Thailand (Bhaibulaya, 1964).²⁸ This fluke requires snails as an intermediate host, and it is not unusual to see man infected with this parasite since snails are included in the diet of the local populace.

Nymphs of Armillifer moniliformis, an arthropod, were found encysted around the mesentery of a rodent (B. indica) intestine. It has been reported before in man but only rarely.

As illustrated in Table 3a, we found four kinds of Amebae and Flagellate protozoa during the survey. All these organisms have been reported in rodents in Thailand before. This laboratory has routinely reported these organisms before from humans and Baughn and Morales

Table 1a: MAP SHOWING RODENT TRAPPING SITES

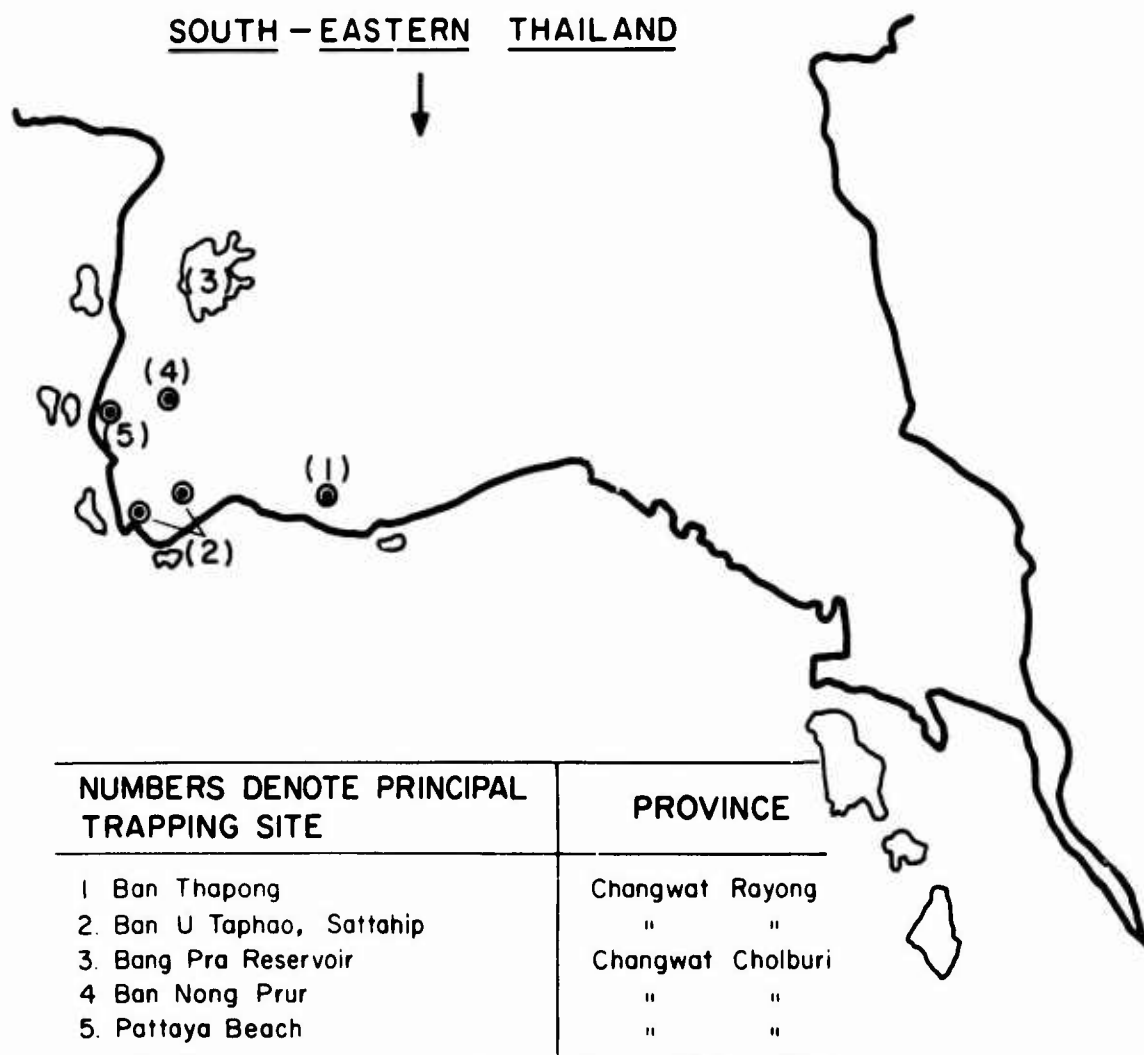


Table 1b: Parasites Found in Rodent Survey

	<u>Genus</u>	<u>Species</u>
Nematodes	Physaloptera	sp.
	Syphacia	obvelata
	Strongyloides	sp.
	Trichuris	muris
	Nippostrongylus	muris
	Rictularia	sp.
	Cyclodontostomum	sp.
	Trichosomoides	crassicauda
Cestodes	Hymanolepis	diminuta
	Hymenolepis	nana
	Raillietina	sp.
Trematodes	Echinostoma	sp.
Acanthocephala	Moniliformis	moniliformis
Protozoa	Entamoeba	coli
	Chilomastix	mesnili
	Giardia	lamblia
	Endolimax	nana
	Trypanosoma	lewisi
	Erythrocytic protozoa	
	Sarcocystis	sp.
Arthropods	Armillifer	moniliformis
Other	Spirochetes	

Table 2a: Results Obtained After Examination of Gastrointestinal Contents

Parasites Found	RAYONG PROVINCE		
	Ban Thapong Area (1)	Utapao-Sattahip Area (2)	
	<u>B. indica</u>	<u>Other</u>	<u>Other</u>
Physaloptera sp.	22.9%	5.0%	26.9%
Syphacia obvelata	4.7%	-	3.1%
Strongyloides larvae	-	-	1.6%
Nippostrongylus muris	-	-	13.7%
Rictularia sp. *	0.2%	3.1%	1.6%
Trichuris muris	15.7%	3.2%	12.8%
Cyclodontostomum sp.	26.4%	2.5%	-
Hymenolepis diminuta	20.2%	5.8%	23.2%
Hymenolepis nana	-	-	0.5%
Raillietina sp.	-	-	1.5%
Echinostoma sp.	0.6%	-	-
Trichosomoides crassicauda	-	-	-
Moniliformis moniliformis	-	-	5.8%
Armillifer moniliformis	-	-	-

* Only ova recovered.

Table 2b: Results Obtained After Examination of Gastrointestinal Contents

Parasites Found	CHOLBURI PROVINCE					
	Bang-Pra Reservoir Area (3)	Ban-Nong Prur Area (4)		Pattaya-Beach Area (5)		
	<u>B. indica</u>	<u>Other</u>	<u>B. indica</u>	<u>Other</u>	<u>B. indica</u>	<u>Other</u>
Physaloptera sp.	5.0%	1.6%	-	2.1%	1.7%	5.0%
Syphacia obvelata	16.7%	0.5%	-	-	1.5%	2.1%
Strongyloides larvae	-	-	-	-	-	-
Nippostrongylus muris	0.2%	-	-	-	-	-
Rictularia sp.	-	-	-	0.5%	-	-
Trichuris muris*	3.3%	0.5%	-	-	-	7.4%
Cyclodontostomum sp.	0.6%	-	-	-	0.4%	1.6%
Hymenolepis diminuta	10.3%	5.0%	-	-	-	-
Hymenolepis nana	-	-	-	0.5%	0.2%	7.4%
Raillietina sp.	-	-	-	-	-	-
Echinostoma sp.	1.9%	-	-	-	-	1.6%
Trichosomoides crassicauda	-	-	-	-	1.0%	0.5%
Moniliformis moniliformis	-	-	-	-	-	-
Armillifer moniliformis	0.2%	-	-	0.5%	-	1.6%

* Only ova recovered.

Table 3a: Protozoans Recovered from Formalin-Ether-Concentrated Fecal Material.

Parasites Found	RAYONG PROVINCE			
	Ban Thapong Area (1)		Utapao-Sattahip Area (2)	
	<u>B. indica</u>	<u>Other</u>	<u>B. indica</u>	<u>Other</u>
Entamoeba coli	8.4%	1.0%	2.1%	8.5%
Chilomastix mesnili	8.8%	2.1%	0.4%	4.8%
Giardia lamblia	-	-	-	-
Endolimax nana	-	-	-	-

Table 3b: Protozoans Recovered from Formalin-Ether-Concentrated Fecal Material.

CHOLBURI PROVINCE						
Parasites Found	Ban-Pra Reservoir Area (3)		Ban-Nong Prur Area (4)		Pattaya-Beach Area (5)	
	<u>B. indica</u>	<u>Other</u>	<u>B. indica</u>	<u>Other</u>	<u>B. indica</u>	<u>Other</u>
Entamoeba coli	3.9%	1.6%	-	1.0%	0.8%	1.6%
Chilomastix mesnili	2.7%	1.0%	-	1.0%	0.2%	0.5%
Giardia lamblia	0.2%	-	-	-	-	-
Endolimax nana	0.4%	-	-	-	-	-

reported all but one of them during their 1971 survey in Thailand. Except for Giardia lamblia all the organisms encountered are commensals, although the finding of these parasites in the rodents clearly implicates their role as reservoir hosts for these parasites.

Only four blood parasites were encountered during our survey. Trypanosoma lewisi as shown in Table 4 was found more in other rodents than in B. indica and is generally a strict parasite of its definitive host. Some mention is made of a girl being infected with this parasite in Malaya, but no description is given of the parasite. A spirochete which can cause a variety of diseases was also found. This parasite could not be identified as to species from blood smears alone. The finding of this parasite in the rodent blood smears is hardly noteworthy, since rat bite fever is caused by a spirochete, and is transmitted by rat bites. Sarcocystis, which is a parasite of tissue, was also found in the blood smears; this organism was considered a contaminant since the blood used to make the smears was taken from rodent muscle tissue. The last organism encountered was an erythrocytic protozoan of unknown species. More work is required before definite identification can be accomplished.

Table 4. Incidence of Blood Parasites in Rodents

	<u>B. indica</u>	Other
<u>Trypanosoma lewisi</u>	1.0%	8.0%
Sarcocystis	19.0%	3.4%
Erythrocytic protozoa	17.4%	0.5%
Spirochetes	0.8%	0.0%

11. Intestinal Parasitism in the Rhesus Monkey.

Studies have been initiated to determine the incidence of intestinal parasites in rhesus monkeys upon arrival from India and to evaluate the efficacy of treatments used to control them.

Over 500 rhesus monkeys per year are needed to support a malaria drug screening program at SMRL. These animals are received in groups of 85 every 60 days, weigh 2-3 kg. upon arrival, and come directly from the wild in India. After a 45 day quarantine period in our colony, they are utilized and then sacrificed within 60-90 days.

In February 1973, a one year study was initiated to monitor the incidence of internal parasites in the rhesus monkeys being received

Table 1

Internal Parasites Found in Rhesus Monkeys

Parasite Found	Before Treatment		After Treatment *	
	Number Positive	Percent Positive	Number Positive	Percent Positive
<u>HELMINTHS</u>				
Ascaris sp.	1	0.6%	1	0.6%
Capillaria sp.	0	0.0%	1	0.6%
Strongyloides sp.	118	69.8%	4	2.4%
Trichostrongylus sp.	57	33.7%	1	0.6%
Ancylostoma sp.	17	10.1%	0	0.0%
Trichuris sp.	7	4.1%	5	3.0%
Unidentified	0	0.0%	3	1.8%
<u>PROTOZOANS</u>				
Balantidium coli	60	35.5%	32	19.0%
Chilomastix mesnili	1	0.6%	3	1.8%
Entamoeba coli	109	64.5%	58	34.5%
Entamoeba histolytica	37	21.9%	37	22.0%
Endolimax nana	25	14.8%	21	12.5%
Giardia lamblia	0	0.0%	15	8.9%
Iodamoeba butschlii	53	31.4%	12	7.1%
No Parasites or Ova	3	1.8%	59	35.1%

* Treatment was 100 mg/kg Thiabendazole via nasogastric tube

from India. Fecal specimens are being taken from each animal on the day of arrival and are examined by the flotation method for ova and parasites. The animals are then treated as a group on the next day. Fecal specimens are taken ten days after treatment and again examined for ova and parasites. Results of these examinations are shown in Table 1.

The data presented in Table 1 represents two groups of 85 animals each, which were received in February and March 1973. It appears from this data that treatment with 100 mg/kg of Thiabendazole is quite effective against all helminths encountered except whipworms, but no firm conclusions may be drawn at this time. When completed in February 1974, this study should give an indication of the internal parasites to be expected in newly arrived young rhesus monkeys from India, as well as an indication of seasonal variation. It should also help in evaluating the parasite treatment regimens being used.

12. Chemotherapy of Gnathostomiasis.

The effect of oral or parenteral administration of drugs on Gnathostoma spinigerum larvae is being studied on a continuing basis. The drugs selected for the screening tests have been used effectively in the treatment of certain helminthic diseases but have yet to be used in treating gnathostome infection.

Experimental white mice and/or white rats were orally infected with advanced third stage larvae of G. spinigerum. Mice were infected with 5 larvae, and rats with 20. The drug or combination of drugs tested was administered orally or by intramuscular injection, and the daily dose was calculated using the body weight of the test animal. In all instances the control animals were given distilled water. The drugs tested were Hetrazan (Diethylcarbamazine citrate) combined with Bithionol (Bitin); Fouadin (Pentasodium antimony 1, 1, 1-biscatechol-3, 5-disulfonate); Flagyl (1-B-hydroxyethyl-1, 2-methyl-5-nitroimidazole); Dehydroemetine (racemic 2-dehydroemetine dihydrochloride); a combination of Dehydroemetine and Flagyl; and Metrifonate (O, O-dimethylhydroxy-2, 2, 2-trichlorethyl-phosphonate). Following infection and the chemotherapeutic regimen, the test animals were autopsied, the parasites counted, and the results recorded.

Hetrazan combined with Bitin (oral administration): Infected mice were treated with 15 daily doses of Hetrazan (10mg/kg body weight) combined with Bitin (50 mg/kg body weight). The results of this experiment are shown in Table 1. No reduction in the number of larvae in treated mice was observed, and this combination of drugs is judged ineffective.

Table 1
Hetrazan/Bitin Chemotherapy on Mice Infected with Gnathostoma spinigerum

Daily dose of Hetrazan (10 mg/kg body weight) combined with Bitin (50 mg/kg body weight) / mouse 2	No. mice treated	Autopsy findings			Remarks
		No. mice positive with advanced third stage larvae	No. advanced third stage larvae found (%)	Organs infected	
15	32	31 (97)	102 (64)	liver and/or body muscles	Autopsies 2-47 days after the last dose.
Control - 15 doses	9	9 (100)	29 (64)	" "	" "

- 1 Drug dissolved in distilled water.
2 Control was distilled water.

Table 2

Fouadin Chemotherapy on Rats or Mice Infected with Gnathostoma spinigerum

Daily dose of Fouadin/rat or mouse 1,2	No. rats or mice treated	Autopsy findings			Remarks
		No. rats or mice positive with advanced third stage larvae (%)	No. advanced third stage larvae found (%)	Organs infected	
<u>(3.15 mg/kg body weight)</u>					
15	12 rats	12 rats (100)	85 (35)	body muscles	Autopsies 17 days after the last dose
Control - 15 doses	3 rats	3 rats (100)	20 (33)	" "	" "
<u>(6.3 mg/kg body weight)</u>					
14	33 mice	33 mice (100)	112 (68)	liver and/or body muscles	Autopsies 14-16 days after the last dose
Control - 14 doses	15 mice	15 mice (100)	45 (60)	" "	" "
<u>(100 mg/kg body weight)</u>					
14	20 mice	20 mice (100)	73 (73)	" "	Autopsies 10-18 days after the last dose
20	20 mice	20 mice (100)	70 (70)	" "	Autopsies 3-26 days after the last dose
Control - 20 doses	10 mice	10 mice (100)	39 (78)	" "	Autopsies 19 days after the last dose
<u>(200 mg/kg body weight)</u>					
14	20 mice	20 mice (100)	69 (69)	" "	Autopsies 17-19 days after the last dose
Control - 14 doses	10 mice	10 mice (100)	35 (70)	" "	" "
20	20 mice	20 mice (100)	70 (70)	" "	Autopsies 10-18 days after the last dose
Control - 20 doses	10 mice	10 mice (100)	39 (78)	" "	Autopsies 2-20 days after the last dose

1 Drug was dissolved in distilled water.

2 Control was distilled water.

Table 3

Flagyl Chemotherapy on Mice Infected with *Gnathostoma spinigerum*¹

Daily dose of Flagyl (200-1600 mg/kg body weight)/ mouse	No. mice treated	Autopsy findings		Remarks
		No. mice positive with advanced third stage larvae (%)	No. advanced third stage larvae found (%)	
(200 mg/kg body weight)				
5	20	20 (100)	72 (72)	Autopsies 16-25 days after the last dose
Control - 5 doses	10	10 (100)	35 (70)	Autopsies 25 days after the last dose
(400 mg/kg body weight)				
5	17	17 (100)	54 (64)	Autopsies 18 days after the last dose
Control - 5 doses	10	10 (100)	37 (74)	Autopsies 16-17 days after the last dose
(800 mg/kg body weight)				
15	19	19 (100)	50 (53)	Autopsies 1-18 days after the last dose
Control - 15 doses	10	10 (100)	29 (58)	Autopsies 18 days after the last dose
(1600 mg/kg body weight)				
15	16	16 (100)	51 (64)	Autopsies 2-20 days after the last dose
Control - 15 doses	10	10 (100)	31 (62)	Autopsies 21 days after the last dose

1 Drug was dissolved in distilled water.

2 Control was distilled water.

Table 4
Dehydroemetine Chemotherapy on Mice Infected with Gnathostoma spinigerum

Daily dose of Dehydroemetine/ mouse 1,2	No. mice treated	Autopsy findings			Remarks
		No. mice positive with advanced third stage larvae (%)	No. advanced third stage larvae found (%)	Organs infected	
<u>(10 mg/kg body weight)</u> 15 Control - 15 doses	19	19 (100)	57 (60)	liver and/or body muscles	Autopsies 3-19 days after the last dose
	10	10 (100)	30 (60)	" "	Autopsies 23 days after the last dose
<u>(15 mg/kg body weight)</u> 15 Control - 15 doses	11	11 (100)	44 (80)	" "	Autopsies 2-19 days after the last dose
	10	10 (100)	32 (64)	" "	Autopsies 2-23 days after the last dose

- 1 Drug dissolved in distilled water.
2 Control was distilled water.

Table 5
Dehydroemetine/Flagyl Chemotherapy on Mice Infected with Gnathostoma spinigerum

Daily dose of Dehydroemetine (10 mg/kg body weight) com- bined with Flagyl (200 mg/kg body weight)/ mouse ^{1,2}	No. mice treated	Autopsy findings			Remarks
		No. mice positive with advanced third stage larvae (%)	No. advanced third stage larvae found (%)	Organs infected	
20	18	18 (100)	62 (69)	liver and/or body muscles	Autopsies 2-36 days after the last dose
Control - 20 doses	10	10 (100)	31 (62)	" "	Autopsies 36 days after the last dose

1 Drug dissolved in distilled water.

2 Control was distilled water.

Fouadin (intramuscular injection): The compound contains 13.5 per cent trivalent antimony in pyrogen-free redistilled water. Both mice and rats were used and a daily dose of drug administered at levels of 3.15, 6.3, 100, and 200 mg/kg body weight. The drug has been used in treating infection with Schistoma hematobium. The results of this experiment are shown in Table 2. No reduction in the number of larvae in treated mice was observed and this drug is judged ineffective.

Flagyl (oral administration): This drug has been used with satisfactory results in treating intestinal and hepatic amebiasis, trichomoniasis and some other protozoal infections in man. Recently this drug has been used with good results in treating dracunculiasis in man.^{29,30} In this experiment, a daily dose of flagyl was administered to gnathostome-infected mice at levels of 200, 400, 800, and 1600 mg/kg body weight. The results are shown in Table 3. No reduction in the number of larvae in treated mice was observed and this drug is judged ineffective.

Dehydroemetine (oral administration): This compound is chemically related to natural emetine and obtained by total synthesis. The drug has been used with good results in intestinal amebiasis (all forms), hepatic amebiasis, schistosomiasis (S. haematobium or S. mansoni or mixed infection) and distomiasis due to Fasciola hepatica. In this study, gnathostome-infected mice were each treated with 15 daily doses of dehydroemetine administered at levels of 10 or 15 mg/kg body weight. The results are shown in Table 4. There was no reduction in the number of larvae in treated mice and this drug is judged ineffective.

Dehydroemetine combined with Flagyl (oral administration): Infected mice were treated with 20 daily doses of dehydroemetine (10 mg/kg body weight) combined with Flagyl (200 mg/kg body weight). The results of this experiment are shown in Table 5. No reduction in the number of larvae in treated mice was observed and this drug is judged ineffective.

Metrifonate (oral administration): It has been reported that metrifonate was an effective drug in the treatment of Schistosoma haematobium infection by oral administration.³¹ This drug is now being studied to determine its effectiveness at 2-week intervals for 5 doses in G. spinigerum infected experimental mice. The doses are 20, 40, 80, and 120 mg/kg body weight. Results at this time are incomplete.

13. Studies of New Experimental Intermediate and Paratenic Hosts and Modes of Transmission of Gnathostoma spinigerum and G. hispidum.

These experiments are a continuation of studies to locate new experimental host animals susceptible to Gnathostoma spinigerum and G. hispidum as reported previously.⁹

Young as well as adult fresh water prawns (Macrobrachium rosebergi, De Mann) were obtained from the Chao Phraya river, ponds in and around the Bangkok area and from the experimental shrimp farm of Department of Fisheries, for testing as possible intermediate and/or paratenic hosts of G. spinigerum. One hundred young shrimp and approximately 20,000 G. spinigerum newly hatched first stage larvae were placed together in a two liter glass beaker containing about 1.8 liters of fresh water. In addition 100 young shrimp were placed in another 2 liter glass beaker with 1000 G. spinigerum fully developed larvae (early third stage) in cyclops.

To test the possibility of fresh water shrimp and prawns being a paratenic host, advanced third stage G. spinigerum larvae obtained from experimentally infected laboratory white mice will be used to attempt infection. Adult shrimp and prawns are being kept in laboratory aquaria for a period of time to familiarize them with the laboratory environment and food before commencing the experiment. In order to determine the possible presence of natural infection, 100 young shrimp and 5 adult shrimp and prawns, obtained from the same source as the experimental crayfish, were autopsied for the presence of gnathostomes. The study on G. hispidum was not done during this reporting period because of time limitation.

The experiments are still in progress. The result of autopsies to date to determine natural infection of shrimp and prawns with gnathostomes is negative.

Project 3A062110A831 TROPICAL MEDICINE

Task 00, Tropical Medicine

Work Unit 002 Tropical and subtropical military medical research

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Project 3A062110A831 TROPICAL MEDICINE

Task 00, Tropical Medicine

Work Unit 002 Tropical and subtropical military medical research

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1. Laboratory Animal Disease in Thailand: Its Occurrence and Importance to Comparative Medicine.

The objective of this study is to detect and investigate spontaneous diseases of laboratory animals. This knowledge will aid in defining and improving the health of laboratory animals maintained in Thailand, and in developing animal models for human diseases. In order to accomplish the objective, a program of continuous surveillance of the health status of the animal colony has been developed. Four areas are emphasized in this program, (1) the disease screening program conducted in the laboratory animal breeding colony, (2) the recurring clinical and laboratory examination of animals housed in the laboratory including those procedures performed during the quarantine of newly purchased animals, (3) the post-mortem examination of each animal that dies in the colony, and (4) the development of standards for operation and quality control. When indicated by the findings, experimental studies are initiated to explore in detail the problems that occur.

There was little evidence of spontaneous disease among laboratory rodents during this report period. The annual production of rats, mice, and guinea pigs has been maintained at levels comparable to previous years, as have indicators of production efficiency such as conception rate and yield per female. Production was lowest during the hot season as in previous years. The number of rodents

necropsied and the distribution of gross pathological lesions according to organ system is shown in Table 1. The most frequently observed gross lesions were lobar pneumonia, regional enteritis, and cystic ovaries. Bacteria isolated from the lungs or feces of mice, hamsters, and guinea pigs as part of the disease screening program are shown in Table 2. The coliform group was most frequent-
1/ isolated, followed in frequency by Pseudomonas sp.

A number of spontaneous deaths occurred among primates in the laboratory during the year. The cause and frequency of deaths in rhesus monkeys during the initial 45 day quarantine period after arrival in the colony are summarized in Table 3. Rhesus monkeys arrive directly from the wild in India, and over 85 per cent of them have intestinal parasites upon arrival. These animals are stressed by capture, transportation, and adjustment to a different environment, and are quite susceptible to enteritis which is often complicated by secondary bacterial infection. This bacterial enteritis accounts for most of the losses due to intestinal symptoms. Most of the losses due to pulmonary disease were attributed to primary measles virus infection with secondary bacterial complications. This was determined from clinical signs and confirmed by observation of pathological lesions of interstitial and giant cell pneumonia. Virus isolation has not been attempted on these animals. There were no cases of tuberculosis diagnosed either by intrapalpebral inoculation with KOT or by post-mortem examination.

Nine deaths occurred among gibbons during this report period. Most of these deaths were due to gastrointestinal conditions caused by a combination of parasitic and bacterial infections, and by respiratory problems. Two cases of granulocytic leukemia developed in gibbons during the year and are reported elsewhere.

Table 1

Summary of Rodent Breeding Colony Pathologic Findings for 1972

Species	Number Examined	Pulmonary	Gastrointestinal Pathology	Genitourinary Pathology
Guinea Pig	122	17	9	9
Mouse	235	10	3	0
Hamster	182	8	2	1

Table 2

Bacterial Isolates Identified in Laboratory Rodents 1972

Bacterial Isolate	Species Examined		
	Mouse (234)	Hamster (177)	Guinea Pig (119)
<i>Staphylococcus aureus</i>	7 (3%)	6 (3.4%)	4 (3.4%)
<i>Staphylococcus epidermidis</i>	0	12 (6.8%)	0
<i>Micrococcus</i> sp.	2 (0.85%)	1 (0.56%)	2 (1.7%)
<i>Escherichia coli</i>	55 (23.5%)	13 (7.3%)	20 (16.8%)
<i>Paracolobactrum coliforme</i>	6 (2.6%)	6 (3.4%)	0
<i>Paracolobactrum intermedium</i>	2 (0.85%)	0	2 (1.7%)
<i>Paracolobactrum aerogenoides</i>	18 (7.7%)	6 (3.4%)	22 (18.5%)
<i>Intermedium coliforme</i>	0	2 (1.1%)	0
Non-hemolytic streptococci	0	2 (1.1%)	0
Alpha-hemolytic streptococci	0	3 (1.7%)	2 (1.7%)
<i>Pseudomonas aeruginosa</i>	0	0	6 (5%)
<i>Pseudomonas</i> sp.	15 (8.1%)	4 (2.2%)	2 (1.7%)
Diphtheroids	0	0	1 (0.84%)
<i>Proteus mirabilis</i>	18 (7.7%)	0	0
<i>Proteus morganii</i>	0	0	1 (0.84%)
<i>Proteus</i> sp.	8 (3.4%)	25 (14.1%)	0
<i>Bacillus</i> sp.	2 (0.85%)	4 (2.2%)	1 (0.84%)
<i>Herellea</i> sp.	2 (0.85%)	0	0
<i>Mima polymorpha</i> var. <i>oxidans</i>	1 (0.42%)	0	0
<i>Mima</i> sp.	0	0	3 (2.5%)
<i>Citrobacter alkaligenes</i>	1 (0.42%)	0	0
<i>Citrobacter</i> sp.	0	0	1 (0.84%)
<i>Providencia</i> sp.	3 (1.3%)	0	4 (3.4%)
<i>Shigella boydii</i>	0	0	1 (0.84%)
No Pathogens Isolated	102 (43.6%)	114 (64.4%)	61 (51.3%)

Table 3

Rhesus Monkey Losses During The Initial
45 Day Quarantine Period.

Month	Animals Received	Number of Deaths	Intestinal Disease	Pulmonary Disease
February	85	44 (51.8%)	18	26
April	85	6 (7.1%)	3	3
June	85	1 (1.2%)	1	0
August	85	3 (3.5%)	3	0
October	85	5 (5.9%)	3	2
December	85	12 (14.1%)	4	8
Total	510	71 (13.9%)	32	39

Table 4

Summary of Gibbon Necropsy Findings

Principle Pathologic Finding	Number of Animals
Pneumonia	3
Gastroenteritis	3
Meningioencephalitis (Cysticercoid)	1
Granulocytic Leukemia	2

2. Studies on the Growth, Development and Reproduction of Gibbons in Captivity.

A colony of 55 gibbons (Hylobates lar) has been maintained at the SEATO Medical Research Laboratory for use in the medical research projects of the laboratory. An active breeding program has been conducted for the past several years, and 16 young have been produced in the laboratory. Insofar as it is consistent with the other research objectives of the laboratory, observations on various aspects of the growth and development of these laboratory raised animals have been made on a regular basis. Information on the growth, development, and reproduction of gibbons in captivity may be useful in medical research, and in developing methods of breeding and rearing gibbons in a laboratory environment.

During the past year, 5 gibbons were born in the colony. Three of these are presently alive and healthy. One was stillborn, and one died at 5 weeks of age of an acute bronchopneumonia. The birth information for these 5 animals is presented in Table 1. Since 1967, six females have produced all of the 16 young raised in the laboratory. It is of interest to note the period of time elapsing between deliveries of successive young. This information is presented in Table 2. The shortest intervals between delivery of young were in B-4 and B-7. These females each had their third baby only 9.5 months after the previous birth. These data indicate that frequent parturition is possible in captive female gibbons, but that it may take several years in captivity to attain a birth rate approaching one young per year. Adaptation to life in captivity and compatibility with the males in the breeding group play major roles in obtaining frequent pregnancies in the female gibbon.

During 1972, gibbon PC-1, the first gibbon born in our colony on 22 December 1967, began to menstruate. Eversion of the vulva was first noted on 12 July 1972, and daily vaginal swabs were taken from that time until the first menstrual flow was noted on 9 September 1972. Its age at that time was 4 years, 7 months, and 19 days and body weight was 4,600 grams. Subsequent menstrual bleeding was observed on 15 October, 11 November, 16 December, 19 January, 10 February, and 8 March at intervals of 36, 27, 35, 34, 22, and 26 days respectively. On the day that vaginal eversion was noted (12 July 1972), the only decidual teeth remaining were the upper

canines. The left upper canine was shed on 16 August, and the right was loose. The right upper canine was shed a few days later.

The regular program of daily observation and vaginal swabbing in 11 of the female gibbons in the colony was continued during 1972, in order to better characterize the length of the sexual cycle and its variability. The results of these observations are indicated in Table 3. The length of the cycle was usually 19-23 days, but intervals as short as 11 days were observed. In nearly all animals, periods of amenorrhea two or three months in duration or longer were noted. It is believed that these longer periods between successive menstruations are multiples of the basic cycle. S-70 and S-81 were quiescent throughout most of the period.

Table 1

Gibbons born in the SMRL Colony during 1972-1973.

Baby Number	Date of Birth	Parents		Remarks
		Male	Female	
PC-12	10 Mar 72	B-8	B-7	Died 17 Apr 72 of Acute Pneumonia
PC-13	11 Aug 72	S-58	B-11	
PC-14	28 Oct 72	B-12	B-4	
PC-15	22 Dec 72	P-16	B-7	Stillborn
PC-16	1 Jan 73	B-8	B-59	

Table 2

Reproductive Data on Breeding Female
Gibbons in the SMRL Colony

Female Number	Date of Birth of Young			
	1st	2nd	3rd	4th
B-4	12 Dec 67	11 Oct 70 (34) (b)	26 Jul 71 (9.5)	28 Nov 72 (16)
B-6	Mar 69 ^(a)			
B-7	12 Jun 68	10 Mar 72 (45)	22 Dec 72 (9.5)	
B-11	10 May 68	16 Sept 70 (28)	15 Oct 71 (13)	11 Aug 72 (10)
B-37	4 May 71			
B-59	Sep 69 ^(a)	12 Oct 71 (25)	1 Jan 73 (14)	

(a) These births occurred under wild conditions on an island in the gulf of Thailand. The exact date of birth is unknown.

(b) Numbers in parenthesis are the number of months since the previous birth for this female.

Table 3

The Duration of the Sexual Cycle in the Female Gibbon

	Gibbon Identification Number										
	B14S	B66S	B85*	B88	P2	P5	S2	S20	S90	S81	S92
Interval in days between successive cycles	63	22	19	22	24	22	23	23	144	101	20
	26	22	22	20	18	56	21	28	207		11
	26	19	18	43	24	21	24	37			61
	33	20	27	22	18	48	25	30			22
	101	14	21	35	27	38	18	29			23
	21	26	22		21	45	20	22			26
	22	21	29		14	21	18	56			28
	31	19	70		15		20	26			19
	25	21	24		21		26	21			
		23	20		29		21	26			
		23	23		16		19				
		20	19		46		19				
		22	38		14		20				
		44			15		25				
		20					23				
		20					21				

* All gibbons in the study are Hylobates lar, except B-85 which is Hylobates lar pileatus.

3. Observations on the Occurrence, Etiology and Treatment of Diarrheas in Captive Gibbons

The objective of these studies was to determine the causes of diarrhea in captive gibbons and to evaluate methods of treatment.

The information included in this report was collected over a one year period in a colony of 55 gibbons (Hylobates lar, Hylobates lar pileatus, and Hylobates concolor) maintained at the SEATO Medical Research Laboratory. Forty-one of these gibbons were maintained in individual cages within a screened laboratory building. Fourteen were kept outdoors in 9 large breeding cages singly or in pairs. The gibbons were fed commercial primate chow daily, and banana, acacia, and oranges were fed 3 times per week. Chlorinated drinking water was provided ad libitum. All cages were cleaned daily and steamed weekly.

Fecal specimens were collected monthly from each gibbon for parasitological and bacteriological examination. In addition, fecal specimens were collected from gibbons with diarrhea on the first day of illness, and following treatment. Symptomatic treatment was initiated on the first day of diarrhea, and the therapy was modified in accordance with the etiology after the results of the fecal examination were known.

Sixty-five cases of diarrhea were observed in forty-eight of the gibbons during the period of observation. Thirteen animals had episodes of diarrhea more than once. The 65 cases are classified by etiologic category and month of occurrence in Table 1. Protozoans were observed 28 times, accounting for 43.1 percent of the cases, while helminthiasis was observed 27 times, or in 41 percent of the cases. Diarrhea caused by a bacterial organism (Shigella flexneri) was observed in only one case. Under the "unknown" category are those cases in which no sample was obtained (4 cases) or in which no causative organism could be identified (5 cases). Multiple infection with both a protozoan and a helminth was noted in 3 cases occurring in August and in January. The highest incidence of diarrhea was noted in the early part of the hot dry season in February and March.

Table 1

Cases of Diarrhea in Captive Gibbons by Etiology and Month of Occurrence.

Number of Cases of Diarrhea													
Etiology	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Jan	Feb	Total
Helminthic	6	1	3	2	4	-	1	2	2	2	2	2	27
Protozoal	5	2	2	1	2	2	-	1	1	2	5	5	28
Bacterial	-	-	-	-	-	-	-	-	-	-	-	1	1
Unknown	1	1	2	1	1	-	-	-	-	-	1	2	9
Total	12	4	7	4	7	2	1	3	3	4	8	10	65

A further classification of the etiologic agents associated with diarrhea in the gibbon is presented in Table 2. Strongyloides sp. was the most frequently identified organism, accounting for 29.2 percent of the cases. Balantidium coli was the second most common organism isolated, and Entamoeba coli third.

Table 2.

Etiologic Agents Associated with Diarrhea in the Gibbon

Etiologic Agent	Number of Case	Percent of the Total
HELMINTHIC	27	41.5
Strongyloides sp.	19	29.2
Ancylostoma sp.	5	7.7
Trichuris sp.	3	4.6
PROTOZOAL	28	43.1
Balantidium coli	14	21.6
Entamoeba coli	8	12.3
Giardia lamblia	3	4.6
Entamoeba histolytica	2	3.1
Chilomastix mesnili	1	1.5
BACTERIAL	1	1.5
Shigella flexneri	1	1.5
UNKNOWN	9	13.9
TOTAL	65	100.0

The treatments used in the management of diarrhea in the gibbon and their effectiveness are summarized in Table 3. Treatment failures are defined as failure to completely eliminate parasites as determined by a fecal specimen taken at the end of the course of treatment. In these cases the therapeutic regimen was repeated. Reoccurrence is defined as reappearance of parasites at some time during the year following apparently successful treatment. Reoccurrence may be the result of re-infection or of recrudescence of sub-patent parasitism.

Table 3

Therapeutic Management of Diarrhea in the Gibbon

Etiologic Agent	Therapeutic Agent	Treatment Regimen	Treatment Failures Requiring Re-Treatment	Reoccurrence
<u>Strongyloides</u> sp. <u>Trichuris</u> sp.	Thiabendazole (Mintezole ^R)	10 mg/lb. b.i.d. orally for one day	1 of 22 cases	<u>Strongyloidiasis</u> 3 of 19 cases recurred in 3-6 month. <u>Trichuriasis</u> 1 of 3 cases recurred in 8 months.
<u>Balantidium coli</u> <u>Entamoeba coli</u>	Carbarsone plus Tetracycline	125 mg for 10 days orally b.i.d. 50 mg. for 10 days orally b.i.d.	1 of 18 cases	<u>Entamoeba coli</u> 4 of 8 cases recurred within the year
<u>Entamoeba histo-</u> <u>litica</u> <u>Chilomastix</u> <u>mesnili</u> <u>Giardia lamblia</u>	Metronidazole (Flagyl ^R)	150 mg. orally b.i.d. for 10 days	3 of 10 cases	<u>Balantidium coli</u> . 5 of 14 cases recurred within the year.
<u>Ancylostoma</u> sp.	<u>Ancylo</u> ^R <u>lol</u>	0.1 ml/lb. subcutaneously repeated in 14 days	0 of 5 cases	None

The frequent occurrence of diarrheas is a problem in the management of gibbons in captivity. Helminths and protozoan parasites are the principle etiologic agents, with Strongyloides sp., Balantidium coli, and Entamoeba coli accounting for the majority of cases. These diarrheas can be treated successfully in most cases with appropriate regimens of therapeutic drugs, but recrudescence and reinfection are a significant problem requiring regular screening of fecal specimens.

4. Leukemia in the Gibbon

The objective of this study is to determine the incidence of leukemia in the gibbon colony, to characterize clinically and pathologically this disease in the gibbon, and to evaluate its transmissibility.

Gibbons in the SMRL animal colony are screened periodically to detect developing cases of leukemia. Gibbons in which leukemia has been detected are placed under close observation and clinical, hematological, and pathological methods are employed to characterize the development of the disease. Necropsies are done on gibbons that die and tissues from them have been inoculated into gibbons and other laboratory animals to determine if the disease is transmissible.

To date, five cases of granulocytic leukemia have occurred "spontaneously" in the gibbon colony (S-74, S-76, S-86, S-90, and S-93), in addition to the four cases of malignant lymphoma described earlier¹. Cases S-86 and S-93 were not studied in detail. A detailed histological examination was done on the other three cases, and bone marrow from one, S-74, was inoculated into 2 baby gibbons, 2 stump-tailed macaques, mice, hamsters, and guinea pigs. Of the 2 baby gibbons inoculated, one died of a laboratory accident and the other (PC-7) developed leukemia 8 months later. No disease appeared in any of the other animals.

During the report period PC-7 was sacrificed and bone marrow was inoculated into two additional baby gibbons (PC-6, PC-8). Tissue was also examined for the presence of oncogenic viruses. PC-8 subsequently developed leukemia and was sacrificed. Bone marrow was passed to another gibbon (PC-17) and tissue was examined for oncogenic virus. The remaining case of "spontaneous" leukemia (S-76) was also sacrificed, bone marrow was passed to a

young gibbon (PC-10) and tissue was examined for virus. At this time, PC-6, PC-10 and PC-17 remain clinically well.

Virus isolation studies were done by Dr. Thomas Kawakami at the Oncology Laboratory, University of California, Davis. An RNA C-type virus was isolated from every animal studied (S-76, PC-7, PC-8). Sera from most of the gibbons in the colony were examined for the presence of antibody against this virus and many were found to be positive, indicating that the virus is widespread throughout the colony.

A manuscript describing the above events has been submitted for publication.

5. A Report of an Outbreak of Acute Enteritis in Swine with Hepatic Lesions.

The objective of this study was to determine the etiology of an epizootic of acute enteritis in swine with pre-existing hepatic lesions. This was a collaborative investigation between SMRL; the Faculty of Veterinary Medicine, Kasetsart University; and the Tub Kwang Animal Husbandry Section, Kasetsart University.

The Tub Kwang pig farm located in Saraburi province maintains approximately 80 adult breeding sows and 50 young adult swine (40-50 Kg.). The animals are housed in covered open barns with concrete floors. The basic diet consists of a locally grown grain mixture with added vitamin, mineral, and protein supplements. Fresh drinking water is supplied from automatic waterers. In early November 1972 a large number of pigs became sick. Within two weeks 65 animals were sick and 21 died. (Table 1). Clinical signs included a slightly increased temperature, icterus, loss of appetite, depression, lameness, and erythematous and edematous skin lesions.

A complete necropsy was performed on three dead and four sick animals, and tissues were taken for complete histopathologic examination. Specimens of heart blood, lung, stool, brain, and intestine were cultured for both aerobic and anaerobic bacteria. Virus isolation was attempted in tissue culture from stool, liver, kidney, lung, spleen, heart, and brain. Weanling mice were

inoculated with serum and with suspensions of liver, kidney, lung, heart, spleen, and brain and observed for 3 weeks. Specimens of drinking water and food were submitted for bacteriologic, mycologic, and toxicologic evaluation.

Table 1

Swine Epizootic at Tub Kwang

Age	Total Animals	Sick Animals	Morbidity	Deaths	Mortality
<1 month	100	0	0%	0%	0%
1-3 months	200	40	20%	15	38%
3-6 months	50	20	40%	3	15%
Adult females	80	5	6%	3	60%
Total	430	65	15%	21	32%

Virology: No viruses were isolated either in tissue culture or by animal inoculation.

Gross Pathology: The skin, mucous membranes and other tissues were markedly icteric on all animals examined. The mesenteric lymph nodes were enlarged and diffusely hemorrhagic. Lung lesions were observed in all the animals, consisting of reddened and consolidated areas with sticky exudate present in the bronchi. The pericardial sac was edematous around the ventricular area, and in one animal 10 ml of clear fluid was present in the pericardial sac. Gastrointestinal lesions were present in all animals, and varied from one animal showing only redness of about 30 cm. of the serosal surface of the small intestine with normal stool, to another showing gastritis with reddened areas of gastric mucosa and bloody mucus filling over 6 meters of the small intestine. Generalized subcutaneous and intramuscular hemorrhage was found, but is attributed largely to supportive therapy with subcutaneous and intramuscular injections of fluids. The livers of all animals were completely yellow and fatty. The livers were normal in size and shape, but of a slightly fibrous consistency in two of the animals.

Microscopic Pathology: Microscopic pathologic examination revealed moderate to severe chronic diffuse hepatitis, diffuse acute

hemorrhagic enteritis, and severe acute diffuse hemorrhagic lymphadenitis in all animals examined. All animals showed lung lesions varying from acute bronchiolitis to bronchopneumonia and chronic interstitial pneumonia. Two animals showed colitis, and one animal showed acute splenitis and myositis.

Bacteriology: In the sick animals sacrificed for necropsy, examination of brain, lung and stool showed no pathogens or abnormal flora. The anaerobic bacterium Clostridium perfringens was isolated from fresh heart blood of three of the four animals. In the three dead animals examined, Pasteurella multocida was isolated from several tissues. No pathogenic bacteria were isolated from the drinking water.

Feed Analysis: No pathogenic bacteria were isolated from the food, but a variety of fungal organisms were identified, including Rhizopus sp., Candida sp., Penicillium sp., Mucor sp., and Aspergillus sp. The chemical examination revealed the presence of chlorinated hydrocarbon compounds in each item of animal feed (Table 2) and in samples of pig tissues taken at necropsy.

Table 2

Chemical Analysis of Feed

Feed	Chemical Detected	
Corn Meal	DDT	0.5 ppm
Soybean Cake	DDT	0.2 ppm
Fish Meal	Eldrin	6.25 ppm
Meat Scraps	Eldrin	6.43 ppm
Ground Feed	DDT	0.23 ppm

The hepatic lesions appear to have developed before the other lesions observed. These liver lesions are consistent with those observed in a wide variety of toxic conditions, and could have been caused by chronic exposure to chlorinated hydrocarbons both in the feed and by frequent applications of chlordane spray in the treatment of sarcoptic mange. The toxins produced by Aspergillus sp. have also been reported to cause chronic liver lesions like those described.

The acute enteric lesions observed can be attributed to bacterial infection. Clostridium perfringens has been reported to cause an infectious necrotic enteritis in baby pigs, either as a primary entity or secondary to other disease conditions. Pasteurella multocida rarely causes a primary disease in swine, but often develops as a disease secondary to other conditions which cause debilitation and decreased resistance. It occurs as a chronic or sub-acute disease of the lungs, and/or as an acute septicemia, but it can often be isolated from the lungs of swine that show no disease or tissue alteration. The simultaneous existence of pasteurellosis and certain other infectious diseases is not uncommon. In one study, Pasteurella multocida was isolated in pure culture from 44% of 314 swine showing some lung pathology but no symptoms of disease.

Considering the age of the pigs in this outbreak, the gross and microscopic lesions, and the response to treatment, the illness and fatalities were probably due to the intestinal bacteria. It appears that the over-all problem was one of chronic liver toxicity from chemical and/or biological products producing debilitating hepatic lesions which in themselves were not severe enough to produce overt disease, but were contributory in producing an environment for existing opportunistic organisms to establish. Pasteurella multocida and Clostridium perfringens, present in the food and environment, but needing a stressed animal in which to establish themselves, produced the severe acute hemorrhagic necrotizing enteritis and bronchopneumonia which resulted in death for many of the animals. Suckling pigs under one month of age were refractory to the condition described here. They were not exposed to the toxic products in the feed, and they were receiving substantial amounts of maternal antibody through the milk to provide protection against the bacteria involved.

Immediately after the outbreak, animals were medicated with oxytetracycline or penicillin-streptomycin, and the food supply was changed. There have been no further cases of illness or death since that time. Many animals which appeared clinically normal were examined at slaughter and were found to have yellow livers similar to those of the animals described here.

Project 3A062110A831 TROPICAL MEDICINE

Task 00, Tropical Medicine

Work Unit 002 Tropical and subtropical military medical research

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Project 3A062110A831 TROPICAL MEDICINE

Task 00, Tropical Medicine

Work Unit 002 Tropical and subtropical military medical research

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1. Drug Use Reporting by a Sample of Thai Students.

The objectives of this study are:

- a. To provide prevalence and incidence figures on drug use by Thai students.
- b. To determine the general characteristics and knowledge about drugs of the Thai student drug user and non-user.
- c. To provide background data for later work designed to help infer whether there is a relation between Thai drug use and friendship with Americans.
- d. To better understand how certain Thai women view their relations with Americans. This will provide additional information to help interpret other research studying the relation between drug use of these two specific populations.

The present paper describes research which is designed to study the use of drugs by Thais. The purpose of this work is to contribute to the effort to prevent heroin and other drug abuse in the

local population, in order to disrupt the Thai drug use which operates to provide primary support to drug growing, distribution, and American drug use in Thailand. It is the intent of the Department of Army that drug abuse prevention and control be a comprehensive community effort which includes education, law enforcement, and community action. It is hoped that the present research can be utilized by the local community to help make decisions concerning what drug education, prevention, and treatment programs appear to be most relevant.

The topic of study described in this paper is the survey of drug use by the Thai student or partner.

Drug use of the following populations will be surveyed:

a. One school system will be selected which is in proximity to a large American base and presumably in a community which has a large amount of contact with American soldiers.

b. A second school system will be studied in a large city in which there are relatively few Americans. Contact with Americans is presumably low.

c. The third population to be studied is made up of bar-girls and entertainers (partners) from a small town in proximity to two large American military installations. These women are normally in daily contact with U.S. servicemen.

d. The fourth population, juvenile delinquents in the Bangkok area, is small but presumably at high risk for drug use. These will be surveyed at a local juvenile home.

e. The last population to be studied is students attending an upper middle class demonstration school run by Chulalongkorn University. This population is probably more like that which attends the International School of Bangkok (ISB) on socioeconomic variables and acceptance of western values than other school populations in Thailand.

Stratified random sampling will be used to select high school students in Korat and Chiangmai. This will be based on grade, school size, type of school, and type of curriculum. In Korat twenty schools will be selected, and about 40 students per school surveyed. In Chiangmai, about 15 schools will be selected with 50 students per school to be surveyed. Every student age 13 or older attending the

Chulalongkorn Demonstration School will be surveyed (about 400), and every juvenile at the detention home will be surveyed (about 600). The partners will be selected from 300 women who report to a venereal disease health center. Each consecutive woman who reports on a given interview day will be surveyed.

The project was begun on 1 February 1973. Preliminary data necessary for school sampling have been collected in Chiangmai and Korat. Students will be selected and assembled to fill out the questionnaire as soon as summer vacation ends. Data for each population will be tabulated and presented as frequency and per cent counts. This will be done for all subjects, as well as separately for those identified as "drug users" and "non-users." The definition of these terms will await data collection.

In summary, a survey of drug use will be administered to several populations in Thailand. This will provide a large data base to estimate the total amount of drug involvement by students in Thailand. In addition, drug use by local partners who have specified relations with Americans will be studied. This will allow speculations as to possible inter-relations between drug use of the US soldier and Thai drug use.

2. A Survey of Drug Use at International School, Bangkok.

Approximately 900 students at International School, Bangkok, were surveyed to determine the extent of their involvement with drugs, and attitudes and aspirations which might be related to drug use. Drug use reporting by this population is more extensive than that of similar populations surveyed at school in America. Variables relating to drug use parallel those previously reported in the literature. Results of this work will be presented following approval for publication of the manuscript.

3. Prediction of Drug Use in Servicemen.

For a description of the objectives of this study see the Annual Report, April 1971-March 1972.

During this year the first phase of the project was completed. Two hundred and ten soldiers were interviewed. Each filled out the 1200 item questionnaire. From this questionnaire 59 questions were selected which were answered the most differently by the drug

user and non-user. Drug user in terms of this report is any individual who has used a drug besides marijuana 25 times or more, or who has used marijuana daily for at least 30 days with some experimentation with other drugs. All other individuals were considered "non-users."

A discriminant analysis based on the response patterns of the two groups indicates that with a post hoc classification 92% of individuals can be correctly classified as drug users or non-users. Of the 88 drug users 80 were correctly identified, and of the 95 non-users 87 were correctly classified.

The discriminant weights derived from this analysis will now be applied to scores on the 59 variables, which will have been obtained from naive subjects. The naive subjects will be the first 450 individuals entering USARSUPTHAI starting in the Spring of 1973. Classifications will be made (user or non-user) and after one year of following the troops through personal interview and urinalysis tests, each individuals' actual classification will be compared with his predicted classification. This will allow an empirical decision to be made as to the efficacy of this procedure in predicting drug use.

The first phase of the soldier study has been completed. Fifty-one variables which separate the user from the non-user of drugs have been isolated. A discriminant analysis based on these variables correctly classifies 92% of subjects (post hoc). These discriminant weights will now be applied to a new naive sample of soldiers and the mathematical classification will be compared to the clinical classification.

4. Drug Use Reporting by a Population of Thai Partners Working Near U.S. Military Installations.

Drug use and addiction may be costly to the individual and his society. These costs can include disruption in job performance, poorer health and involvement with other crimes. The ready availability of most "illicit" drugs and the high purity, easy availability, and low cost of heroin make this kind of behavior especially dangerous in Thailand.

The number of Thai drug users appears to be increasing, as the total number of people who come to the hospitals for drug treatment

today is many times larger than the numbers reporting several years ago. The total number of heroin addicts in Thailand in 1972 was estimated as over 300,000; there were only 60,000 cases estimated in 1968. It is noteworthy that this number is over 0.8% of the total Thai population (about 36 million).

There is, however, little empirical data available on which to base decisions concerning relevant education, prevention, and treatment programs for drug use in Thailand. There have been no surveys conducted, and no formal research to study the prevalence and incidence of this problem. Few studies of the drug user exist; these have not been conceptually strong. Most data available in Thailand today is based on clinical impressions from individuals working in the drug abuse area, and inferences based on numbers of individuals seeking treatment.

Of particular interest to American authorities in Thailand (military, embassy, etc.) is a determination of the extent to which Thai drug use affects incidence of American drug use. Information bearing on this point could be utilized to suggest decisions concerning more effective ways to control the problem of drug use among Americans in Thailand. Relevant decisions might be whether to allow Americans to live off of a military installation, whether to allow local nationals to utilize special facilities set up primarily for Americans in Thailand, the extent to which cross-cultural friendships should be encouraged, etc. The present study was designed to provide preliminary information on the amount of drug use reported by a specific Thai population, to infer how behavior of this population affects American drug use and to evaluate the feasibility of utilizing the interview technique to study drug use by this population.

Subjects: Four hundred and ninety-eight Thai women were interviewed. (Age range 16-48, average = 26, S.D. = 9.15). Average education was 3.1 years, S.D. = 1.8. These women were primarily bar girls or "hired-wives." A hired-wife is a woman paid to live with a man, and who in addition to being a sexual partner provides services such as house cleaning, cooking, laundry, etc. All women included had reported to the venereal disease Health Center at Kilo Sip, Thailand, to obtain a weekly check-up. An additional 20 women refused to cooperate; no information was collected from them.

Apparatus: A questionnaire designed to assess drug use by the subject, her friends, and U.S. servicemen partners was constructed. This was administered to 30 women and modified. A second revision was administered to 100 women and modified again to provide the final form of the questionnaire. Twenty-two study items were included, in addition to five "lead in" questions and five decoy questions. In addition, a ten item questionnaire dealing only with medical support and physical health questions was included.

Procedure: All women were interviewed in Thai by public health nurses from the South East Asia Treaty Organization Medical Research Laboratory. (SMRL). Three nurses conducted all the interviews, with two nurses working on a given interview day. The women were first examined by the chief nurse of the Health Center, and following this she explained to each potential subject that a health survey was being conducted. The chief nurse would then introduce the subject to one of the SMRL nurses conducting interviews. Subjects were then transferred to one of two private offices for interview. Approximately five minutes were devoted to explaining the nature of the study and establishing rapport with the subjects. It was emphasized that this was a medical survey by a medical organization. Each prospective subject was allowed to refuse to answer questions. No names or identification numbers were asked. During this time it was ascertained that the women had not been previously interviewed. The ten medical questions were first asked. Following this the questionnaire from the present study was administered. All questions were orally asked of each respondent. Data were recorded during the interview, and amplified immediately after. Data were collected from January to May 1972.

Tables 1-4 present results based on the 497 subjects included in the study; the numbers in parentheses are actual frequencies. All per cent values are rounded to the nearest whole per cent. Responses to "lead in" and decoy questions are not presented. At the time of the study all subjects solicited only among American servicemen for partners. Individuals were considered "drug users" if they reported daily use of a drug for at least the previous 30 days. The exception to this is alcohol, the use of which had to be daily for the previous 90 days in order to classify a subject as a drug user.

Table 1 presents drug use characteristics for all 82 drug users. Alcohol and marijuana are reported most frequently; little

amphetamine and barbiturate and no heroin or hallucinogen use is reported. Most drug users have utilized the drug more than 8 months and many more than a year. Fifty per cent of the subjects reported their first use was primarily motivated by the suggestion of a Thai friend, and an additional 11% reported an American friend first suggested the drug use.

Table 2 presents data concerning alleged drug use by other Thai individuals living and working in proximity to the respondents. We see that the respondents view many individuals as using some drug, most commonly marijuana or marijuana and barbiturates. Very little heroin, alcohol or amphetamine is reported used. In addition, about 1/3 of all subjects indicate over half of their friends are drug users.

To determine the degree of involvement with Americans respondents were asked if they had a steady American boyfriend. Steady was defined as existing when the women lived with only one partner or restricted herself to going out with one particular American male. Seventy per cent indicated they had a steady boyfriend. Of these 70%, 46% indicated that financial or other tangible advantages were their main reasons for maintaining their relationship. An additional 32% (175) indicated that financial advantages in addition to some emotional involvement were reasons to maintain the relationship, 10% (57) indicated that the sole reason for maintaining their relation was emotional involvement regardless of money, and 12% (67) indicated that neither emotional involvement nor financial benefits were the most important reasons to maintain their relationship.

Table 3 presents the reported drug use of the individuals with whom these Thai women maintain these relationships. A large percentage of "previous" boyfriends were reported to have used drugs. Marijuana is the drug most frequently reported used. Among present boyfriends a much smaller percentage allegedly use drugs. Again, marijuana is the drug most frequently reported used.

Ninety-eight per cent of all women said they do not like American boyfriends who use drugs. Table 4 presents some of the attitudes and influences which relate to drug use by the respondents' boyfriends and to the respondents themselves. Two per cent indicated that they encouraged their American boyfriends to use drugs. Fifty-two per cent indicated that they tried to stop their

American boyfriends from using drugs. Few Americans are perceived as following the suggestions of these Thai women. It is interesting to note that 48% of the women report that their boyfriends have encouraged drug use but at least 70% refused to follow these suggestions.

Those individuals who indicated that they do try to stop their American boyfriends from using drugs indicated using the following methods: "ask him to stop" 93% (241), "refuse sex" 5% (12), "refuse to buy drugs for him" 1% (1), "keep drugs away from him" 1% (1). Four individuals did not answer. The drugs of which the American is reported to encourage use are marijuana (87%), marijuana and some other drug (8%), alcohol, heroin, or barbiturate (5%).

Fifteen per cent (82) of the women in this study reported current use of some drug. Again, current use was daily use for the previous 30 days. Half of the women reported alcohol as the drug being used and about 1/4 reported using marijuana (see Table 1). Barbiturates were reported used by another 10% of subjects. It is apparent that this represents relatively long-term behavior. Sixty per cent of subjects indicated using the drug at least a year and only 10% for three months or less. There was no current multiple drug use reported.

Thai friends were listed as the most common source suggesting first use of the drug, but 11% of the women indicated that an American friend was the agent first suggesting drug use. As will be seen later this drug was probably marijuana.

Self report of drug use is considerably lower than that reportedly alleged to others (see Table 2). Almost 60% of the women indicated there were many drug users in the area. The definition of "many" was left up to the respondents. Marijuana and barbiturate use account for 90% of the drugs reported used by "other" people, with some heroin use. Surprisingly, alcohol is reported used by only 1% of respondents. Thus, results in Table 1 appear inconsistent with those of Table 2. It is possible that although many individuals report daily alcohol use, they are not intoxicated and therefore are not viewed as "drug users" despite the definition furnished to the respondents.

It is apparent that the women are aware of the use of drugs, as 30% report over half of their friends use some drug daily. One might argue that drug use by a few people could be a "high visibility" phenomenon and the low self use but high "other people" use reported is possibly explained by this (i.e., many people view the same few drug users). The authors feel that this is better explained as a deliberate attempt by individuals to conceal illegal behavior. During the time of this study there were several rumors predicting police action to suppress drug use. In addition, heroin was reported used by "others" but not by the individual respondents. Several women, although obviously intoxicated on a sedative-like drug during the interview, denied drug use. When faced with this fact, they admitted to barbiturate use. Informal observation of this population by the authors also suggests that many more women used sedative-like drugs than the number reported here. Other indirect evidence to corroborate this (such as deaths, hospital admissions for overdose, etc.), are lacking.

A surprising 70% of the women indicated having a steady American boyfriend. This is unusual in that there should then be little reason to seek weekly V.D. checks. This could be explained as: a) exaggeration, as having a steady boyfriend carries more status, b) a matter of convenience, as some of the local night clubs required a current V.D. check for entrance, or c) since not all women who reported having a steady boyfriend actually lived with him, this may reflect mistrust on the part of the American who requires the respondent to be checked.

From Table 3 it is apparent that many boyfriends use drugs, according to respondent report. Marijuana accounts for over 60% of this drug use. Relatively few Americans are reported to use some drug without also using marijuana. This is consistent with many other studies of drug use which report most drug users have used marijuana.

Perhaps the most interesting aspect of Table 3 is the low drug use reported for current boyfriends vs previous boyfriends. This could be due to making comparisons between cumulative past experience and current point incidence, or it might be an attempt to hide illegal behavior of the present boyfriend. It is certainly to the women's best advantage not to disclose this information. About 1/3 of this American drug taking is alleged to occur daily.

The Questions presented in Table 4 were included to offer information on possible influences from one population on drug use of the other. Few Thai women report encouraging drug use by Americans; most report discouraging drug use; and most women view these efforts as fruitless. Since replacement of a girl friend is a simple matter, it should be expected that about the only method utilized to influence American drug use was to ask him to stop using drugs. As indicated in Table 4, few women refused sex or refused to buy drugs for the boyfriend. Table 4 also indicates that 48% of women report that their American boyfriend has encouraged drug use. This is probably consistent with the earlier statement that in 11% of cases an American introduced drug use. Although 71% of the prostitutes indicate they do not follow the suggestion, 29% sometimes do. Eighty-seven per cent of the time the drug use encouraged concerns marijuana. This is consistent with informal observations of soldiers and their Thai girl friends. This may indicate that there is some influence by one population on drug use of the other. The results presented here, if valid, would seem to indicate the effect is primarily to increase marijuana use by the Thai women.

The authors are uncertain as to how to interpret these results. As is apparent from the earlier discussion (e.g., comparison of Tables 1 and 2, and information within Table 3), there is evidence that the women are not reporting their own drug use honestly. The examining nurses also noted a consistent posture of boredom or lack of interest in the latter part of many of the interviews. This suggests that a different research strategy should be employed to collect valid drug use data from this population. This could be: a) an anonymous, self-administered questionnaire (probably impractical), b) case study of a relatively small number of women (to establish better rapport, and to include more personal observation) or c) limiting questions to recent behavior (but not present) or to "what are others doing?".

It is apparent, however, that these women represent a population which is "high risk" for drug use. This is based both on self report (which is probably too low) and inference based on what "other people" allegedly use. It seems that this population is one which could benefit from government efforts to prevent drug use. But since prostitution is illegal, as well as drug use, any outreach program directed at this population would face many problems.

TABLE 4
Description of Drug Use for Those 82 Subjects
Reporting Current Drug Use

Drugs Used	Duration of Drug Use	First Use Suggested by
Alcohol 49%(40)	More than 1 yr. 43%(35)	Thai friend 49%(40)
Marijuana 22%(18)	About 1 yr. 16%(13)	Drug store 16%(13)
Barbiturate 12%(10)	More than 3 mths. 32%(26)	Amer. friend 11%(9)
Amphetamine 9%(7)	Less than 3 mths. 10%(8)	Other source 24%(20)
Other combinations 8%(7)		

TABLE 2
Perceived Drug Use of Others as Reported by Respondents

Are there many people who use drugs everyday around?	Drugs reported used	How many of your friends use drugs everyday?
Yes 59%(293)	Marijuana and barbiturate 51%(148)	Over half 31%(153)
No 24%(120)	Marijuana 28%(82)	Some 17%(84)
Can't say 16%(80)	Barbiturate 12%(33)	None 6%(31)
	Heroin & MJ or Barbiturate 7%(19)	Don't know 46%(225)
	Heroin 1%(3)	
	Alcohol 1%(2)	
	Amphetamine 1%(2)	

TABLE 3
Drug Use by Respondents' American Partners

	Does Boyfriend Use?	Drugs Used	How Often
Previous			
Yes	42% (208)	Marijuana 65% (135)	
No	58% (289)	Marijuana plus some other drugs 21% (44)	
		Amphetamine 3% (5)	
		Heroin 3% (5)	
		Barbiturate 2% (4)	
		Alcohol 1% (2)	
		Other combinations 6% (13)	
Present			
Yes	19% (92)	Marijuana 62% (57)	Daily 36% (33)
No	81% (405)	Marijuana plus some other drugs 19% (17)	More than once/wk 3% (3)
		Heroin 9% (8)	Don't know 48% (45)
		Alcohol 4% (4)	Refuse answer 12% (11)
		Barbiturate 3% (3)	

TABLE 4

Attitudes and Influences Relating to Drug Use

	Encourage American Boyfriends to Use Drugs?	Try to Stop Them?	Does He Follow Suggestion?	Does American Encourage You?	Do You Follow Suggestion?
Yes	2%(10)	52%(259)	6%(17)	48%(238)	3%(6)
No	98%(483)	48%(234)	75%(194)	52%(254)	71%(168)
Sometimes			17%(44)		27%(64)
Don't know			2%(4)		

5. Perceived Distance from Father and Its Relation to Drug Use in a Soldier Population

This study is being conducted to determine if reported distant relations with one's father is associated with use of drugs, or if it is associated with general psychiatric problems including use of drugs.

Recent research conducted at the U.S. Army Hospital, Bangkok, has indicated that soldiers who are treated for drug use problems seem to have a markedly different perception of their relations with their parents (especially their father) than soldiers who have not been involved with drugs. Approximately 50 questions dealing with soldiers' relations to their parents had been asked of drug users and non-users in a recent study. As Kojak predicted, responses to these questions indicated that the user of drugs is more distant in his relations to his father than is the non-user. No such differences were discovered which related to closeness to their mothers. In psychiatric terms, this would probably be described as ambiguity in the individual's relations to his father or authority.

In the present study the questions described above (father distance scale) are being administered to four populations. These populations are made up of: 1) drug users, 2) suspected drug users ("experimenters"), 3) psychiatric patients who do not use drugs, and 4) non-users of drugs. It is anticipated that the drug user would be most different from the non-user, and would be relatively similar to the suspected drug user or "experimental" user.

In addition, the authors also feel that the person with behavioral or psychiatric disorders not involving drugs is likely to be similar to the user of drugs on a variety of measure including relations with his father. Thus, drug use is viewed as but one of several deviant behaviors which may arise at least in part from some of the same etiologic factors. The etiologic factor in this case is an abnormal development of relations with parents, specifically the father.

The purpose of this study is to further aid in the understanding of the drug user and the psychodynamics of drug use. It is expected that this will contribute to the most efficacious medical decision as to the treatment approach to be utilized for these

patients. Specifically, how much time should be devoted to coping with the patient's apparent conflicts in his relations to his father or authority. It is also felt that the father distance scale might be used to identify or "triage" patients who might be high risk for drug use. Thus, those who more closely resemble the drug dependent individual might be more closely followed, with extra urinalyses and psychiatric supervision. But, individuals who more closely represent non-users would be less a matter of current concern to the psychiatric facility.

Subjects: A total of 100 subjects (through age 26 and rank E-6) divided into four equal groups will be utilized. The groups are as follows:

a. Drug Users: These are individuals who have used any drug besides marijuana or alcohol at least 30 times in the last 45 days, or who had been admitted to the hospital for the second time due to the use of some drug besides alcohol or marijuana at any level of use.

b. Drug Risk: These are individuals who have received a positive urinalysis result but deny using any drug, or individuals who admit to drug use but whose total involvement excluding marijuana is 25 times or less. Alcohol will not be included. For these individuals there is no known continued and repeated involvement with drugs. Additional urine from these individuals is normally tested for presence of drugs.

c. Psychiatric Non-Users: These are individuals who report to the Department of Psychiatry for any psychiatric problem, but who have no involvement with any drug besides occasional marijuana (15 times or less total) or alcohol use.

d. Positive Controls: These are individuals who have a positive urine but who, according to documentation in their medical records, were taking the drug for which the positive occurred on medical advice. These are individuals who are normally considered to be completely free of any drug involvement and are not tested further.

Procedure: Each individual who receives a positive urine must report to the U.S. Army Hospital, Bangkok, as an out-patient for consultation with a physician to determine whether he has been involved with drugs. Every consecutive individual who so reports

will be included in the study until the requisite number of subjects per group has been reached. A nurse will give each potential subject the questionnaire form to be filled out while he waits to see a physician. The form is self-explanatory, no special instructions are needed to complete it, and about five minutes are required for completion. The patient will turn the form in to the doctor who will check to see that the social security number has been included. Forms will be collected on a daily basis from the five physicians who work in the out-patient clinic.

A total score will be determined for each subject. Answers will be scored so that a score of 0 would obtain from a person who answers all questions like a non-user, and a score of 17 would obtain from a person who answers every question like a drug user. A one-factor analysis of variance will be used to determine if overall differences between group means exist. Individual T tests will be utilized to determine the exact location of these differences.

Findings will be interpreted to suggest maximum clinical utility as discussed above. Data collection has begun and it is anticipated that all data will have been collected by 15 June 1973.

6. A Follow-up Study of Japanese Encephalitis in Northern Thailand.

Examination of the medical literature indicates that there are many studies of Japanese encephalitis describing the critical symptoms present in the first 60 days after disease onset. There are, however, few studies which present data on convalescence beyond this acute stage. These latter studies present information collected from 1-15 years after onset of Japanese encephalitis (JE).

The only detailed long term study of JE was done by Goto, who eventually followed 43 cases for 15 years. His work is, however, of limited utility to other physicians. This is because most of his reports have been only in Japanese, and since one cannot derive from his work what definitions were utilized for memory loss, mental impairment, disease severity, etc., the physician cannot utilize this information to aid in decision making and counselling processes.

None of these studies has adequately examined one of the most important effects of JE, that of intellectual impairment. Information

In this area has usually been limited to a general statement indicating lower intellectual performance; none has quantified data either during the acute phase or during convalescence.

In addition, no study (with the exception of that by Goto) which has continued beyond the acute stage has presented evidence for anything but very gross neuropathology, intellectual deficit, or mental status abnormalities.

The importance of age as a determinant of severity of onset and degree of recovery has generally not been considered. Age is important because younger individuals appear to be at higher risk for sequelae than older people. Thus, one is more likely to find severe sequelae in patients below about age 10 to 15.

We feel that one of the most important contributions of research in this area is to provide the physician with information which will help him determine the probable outcome or prognosis of this disease. This would allow him increased efficiency in deciding who, for example, should receive occupational therapy, physical therapy, more intensive follow-up, etc., in order to ensure most complete recovery; and to provide information for the most accurate counselling for family and friends of the patient. Thus, the physician could more adequately describe the probability of a given symptom being present, for example, one year after onset of the disease.

The following studies have responded to these points. Dickerson, et al, did a complete study of U.S. soldiers who contracted JE in 1952 in Korea. He attends to the problem of prognostication and indicates that a long fever or presence of a positive Babinski sign indicated a serious outcome. These symptoms predicted either death or neurological residuals at 10 weeks. No further follow-up was obtained. Goto indicates that individuals who acutely present with hyperkinesis have a more favorable prognosis than those presenting with hypokinesis. Pieper and Kurland report that acute convulsions and neuronal involvement correlate with sequelae ten years later. No specific figures are given.

The present study was designed to more adequately measure intellectual performance and provide greater detail on some of the possible correlates of the severity in JE. Thus, we will closely

consider possible age differences, sex differences, and quantification of intellectual impairment. These will be presented in a manner consistent with maximal prognostic value.

Subjects: One hundred and ninety-six individuals (125 male and 71 female) were initially included. Population characteristics (stability, education level, occupation, etc.), are described in the previous annual report. All were residents of the Chiangmai Valley in northern Thailand who presented to one of the local hospitals with symptoms indicative of possible JE infection. Only those 119 individuals (77 male, 42 female, average age 11.1, S.D. = 7.7), whose diagnosis could be definitely established serologically as Japanese encephalitis and who agreed to cooperate were retained for study. Eleven of the 196 subjects were eliminated as they lived too far away for follow-up, 3 additional patients refused to cooperate, and 8 were definitely diagnosed as non-JE. Fifty-five subjects were eliminated because they were serologically negative for JE, or because they died ($n = 37$) and no serological diagnosis could be made.

Apparatus: Standard forms were constructed to record state of convalescence. These forms were: a) Mental Status - to measure psychiatric aspects of subjects' convalescence; b) Neurological - to measure present neuropathology; c) Physical Examination - to describe physical condition as well as certain neurological conditions; d) Home and School - to record feedback from the family, teacher, or employer on a variety of neuropsychiatric symptoms, and provide direct observation of the subject in these environments; e) EEG - to record electroencephalogram findings.

In addition, hospital records were scrutinized to obtain additional or missing data. Objective psychometric tests were also utilized. Those selected were: a) three sub-tests from the WAIS or WISC (digit span, digit symbol, and block design), and b) the Memory Test (to measure short-term memory). This latter test was developed in Thailand and requires the subject to recall the location of "X" marks on various complicated geometrical designs.

Procedure: Each patient went through an identical sequence of examinations. All record keeping was done on the forms described above. Any patient who reported to the out-patient clinic of one of the five participating hospitals between 10 May and 30 October 1970, and whose clinical picture resembled that of JE was

included in the study. These patients were referred to one of the six participating physicians for a physical examination, blood drawing, and in some cases drawing cerebrospinal fluid (CSF). As soon as the patient's health allowed, he was transported to the one hospital where neurological, mental status, and psychometric examinations were conducted. One of two physicians conducted all neurological examinations, one psychiatrist conducted all mental status examinations, and one of four psychologists conducted psychometric examinations (only patients above seven years of age were so tested). EEG examinations were also conducted. Those patients in which JE could not be confirmed were excluded from the data analysis.

At discharge patients were given appointments to return to the hospital. These return visits were scheduled at 1, 2, 4, 6, 9, and 12 months following discharge. Each visit required one full day of examination. This schedule was followed as closely as practicable. On each return visit blood was drawn and the patient received the same examinations described above.

On the same follow-up schedule, one of two SMRL public health nurses visited patients' homes and schools or places of work. On each of these visits the Home and School form was completed. This was based on information asked of family members, teachers or employers, and from personal examination of the patient. Questions dealt primarily with social adjustment but included observation of neuropathological signs and symptoms.

All comparisons presented in this paper are based on the following eleven clinical variables; these were evaluated each time a subject was examined:

1) Level of Consciousness: recorded as normal as long as the patient was alert or dull. 2) Emotional Behavior: this is a 15-item scale made up of items such as irritability, aggressiveness, restlessness, paranoia, incontinence, and depression. For each item scored as "normal" the subject received a score of 1. Total normal items for each subject were presented as a per cent of the total symptoms on which information was available to score on that subject. Thus, a score of 50% indicates that the subject was normal on 50% of those items on which information was available; the total number of items scored was not always 15. 3) Memory

and Intellect: this is a 6-item scale based primarily on how supervisors rated work and school performance. 4) Convulsions: these were scored as either present or absent. 5) Motor Paralysis: this was scored normal or abnormal. Any paralysis, weakness, loss of muscle strength, gait abnormality, etc., was considered abnormal. 6) Involuntary Movements or Tremor: subjects were scored as normal or abnormal. Abnormal was considered to be the presence of any involuntary movement or tremor. 7) Ataxia: subjects were scored as normal or abnormal. Any ataxia was considered to be abnormal. 8) Tone: subjects were scored as normal or abnormal. Any rigidity, spasticity, or flaccidity was considered abnormal. 9) EEG: subjects were scored as normal or abnormal. 10) Digit Span, Digit Symbol, and Block Design: scores on each were converted to a percentile score based on American norms from the WAIS or WISC. 11) Memory Test: scores were recorded as normal or abnormal based on Thai norms.

Observations from the seven follow-ups are presented for four time zones. The first zone consisted of the first 30 days after disease onset, the next two time zones consisted of 100 days each and the last time zone consisted of all convalescence after 230 days. Any symptom which was abnormal at any time in a time zone was recorded as abnormal. Some subjects may have had two or three observations made during a time zone. For psychometric testing the average score was selected if more than one observation was made. Few subjects were completely omitted for an entire time zone. Most symptom scores were recorded during each follow-up by more than one observer. If either reported abnormality for a symptom, "abnormal" was scored.

The results based on nine clinical variables are presented for younger (age 0-10) and older (age 11 and older) subjects for each of the four time zones in Table 1. Average days before hospitalization for younger patients was 3.3, and for older patients it was 4.0.

Examination of Table 1 indicates that on almost every symptom there is improvement for each age group between time zone I and time zone IV. In addition, we see a consistent pattern of greater improvement for older subjects and a higher level of functioning during the acute phase. The only exceptions to this are for ataxia; younger subjects apparently had less ataxia during the acute phase. On memory and intellect there is an apparent decrement

in performance for both groups following the first time zone.

Psychometric test scores are presented in Tables 2. It is apparent again that there is a consistent trend towards improvement from the first through fourth time zone. It should be noted that scores for the first three tests are based on American norms and are percentile rankings. The average percentile ranking should be 50 for a normal population. The 4th test is based on Thai norms and the average per cent normal for all subjects in each time zone is presented. The first three tests indicate that by Zone IV, subjects were responding relatively close to expected American norms (50%), but on the Memory Test only 44% fall within the normal range based on Thai norms.

Table 3 presents the percentage of subjects who were normal and abnormal in Zone IV as a function of their status in Zone I. Reference to this table indicates that those subjects who were normal during Zone I were more likely to be normal during Zone IV than subjects abnormal during Zone I.

Table 4 presents the percentage of individuals having various numbers of abnormal symptoms during Zone I and IV. Inspection of this table indicates that no individual was completely normal during Zone I. And about 23% of individuals, both younger and older, were completely normal at Zone IV. Older subjects again appear to have fewer symptoms, both at Zone I and Zone IV.

Table 5 presents results of CSF examination. Inspection of this table indicates that there are no differences between younger and older or living and dying subjects on cell count or protein mg%. As all differences were well within one standard error of each other no formal statistical tests were conducted.

The analysis scheme utilized was selected to allow the most efficient presentation of large amounts of data. In a longitudinal study such as this, it is inevitable that some observations will be omitted. To help compensate for this, the period of convalescence was divided into the four time zones described earlier. This usually provided two follow-up visits per time zone, and greatly reduced the number of subjects on whom no observations were made for an entire time zone. It should be noted that observations could have occurred at any time during a given zone. The use of "per cent normal" for the multiple item "symptoms" (emotional behavior, and memory and intellect) also helped

eliminate missing data due to inapplicability of this for preschool populations. The symptoms were scored only on the basis of the home visit for these younger patients.

The symptoms presented (with the exception of EEG and psychometric testing) were all recorded by at least two observers during the hospital or home visits. If any observer recorded an abnormality, the symptom in question was recorded abnormal. This is to ensure that no abnormality was omitted. No attempt was made to assess inter-rater reliability.

The Japanese investigators first suggested age effects, reporting the same incidence of infection in younger and older subjects (older were 16 years of age), but more severe sequelae in the younger patients. Hullingshorst, et al., found that 63% of his Korean patients were 2 to 12 years old. Pieper and Kurland report relatively higher incidence of sequelae in children age 0-9 than in older subjects. Present results indicate that the older patient will have, on the average, a less severe onset and is likely to have a more complete recovery. These results are presented in Table 1. Level of consciousness was analyzed as an acute symptom only; no subject-evidenced disturbance in this area lasted longer than 30 days.

The symptom which samples the greatest variety of behavior is "emotional behavior." The items selected for inclusion in this scale were chosen from several of our forms and cover mental status and social adjustment. We see that even after 230 days individuals are, on the average, normal on less than 75% of the areas included in this symptom complex.

The "memory and intellect" symptom complex was made up of six items which consisted mostly of work and school performance. Again, we see that by the fourth time period individuals average less than 75% normal for the areas measured. The large Zone I values here are probably an artifact, as those patients who were too sick to go to school or work during Zone I were not measured. The remaining subjects (the most healthy) remained. By Zone II when the most severely ill patients could be included for measurement, the averages for the groups become much lower.

It is apparent that most of the symptoms indicating neuronal damage do not show a great amount of abnormality. One striking

finding was the large percentage of abnormal EEG responses recorded even after 230 days. This is especially true for the younger patients, for whom only 35% normal EEG's were reported. This might indicate a great deal of residual brain damage which is not directly related to the other symptoms measured, or brain damage which has been compensated.

Scores on the psychometric tests (See Table 2) generally do reflect a decrement in memory and visual-motor performance. American norms were utilized to score the digit span, digit symbol and block design. From these norms we calculated a percentile ranking. We see that on the digit span this value has been exceeded but on the digit symbol and block design it has not quite been reached. On both the digit symbol and block design scores appear to be still rising. These tests should all be sensitive to memory loss, and the latter two (digit symbol and block design) sensitive to difficulties with visual motor coordination. On the basis of these tests, we conclude that average memory loss is not great. It is possible that these scores are all biased in an unknown direction due to the utilization of American norms; no Thai norms exist.

Thai norms for each age have been established for the Memory Test. We see that by Zone III Memory Test scores have levelled off at only about 44% normal. Thus, 56% are below normal. This is a quite different result from the other three tests, and is perhaps more consistent with the findings presented in Tables 1 and 2. We conclude that memory loss is a reliable effect of JE. This has been consistently reported in the literature, but has never been quantitatively measured.

It is apparent that a consistent pattern of greater disease severity for younger patients has emerged. This cannot be ascribed to time of hospitalization (younger patients were hospitalized earlier, which is consistent with a more severe disease onset) or differences in supportive care (18 younger patients and only 5 older patients received physical therapy). One might speculate that these differences might reflect different virulence of the infecting agent, due to older individuals having developed partial immunity after prior exposure.

Table 3 allows determination of the probability that a particular symptom will be abnormal after approximately 3/4 of a year of convalescence. We can see that most people who have a symptom

abnormal during Zone I recover by Zone IV. Relatively few cases exist in which a symptom was recorded as normal during Zone I but abnormal during Zone IV, and these few probably represent individuals who were too ill to be adequately evaluated during Zone I. For example, due to the generalized weakness that accompanies JE, motor paralysis might easily be overlooked until the patient's strength returned. This table can be utilized to help predict patients' later convalescent status, based on the acute (Zone I) level of functioning.

Table 4 provides a general impression of the overall severity of the disease. It is based on a count of symptoms in Table 1 which were abnormal (excluding emotional behavior and memory and intellect) and including the Memory Test. Every subject had some abnormality during the first time zone; most subjects had between three and five symptoms abnormal. By the fourth time zone 23% of younger and older subjects are normal, and there has been a shift to having fewer abnormal symptoms for the remaining subjects. Again, it appears that older subjects have fewer numbers of abnormal symptoms than younger subjects. Goto reported that about 75% of his subjects still had sequelae after three years (both young and old). Simpson and Meiklejohn report about 30% of their patients had some neurological sequelae one year after infection. However, they were able to follow only 1/3 of their original sample. The degree of behavioral impairment that these sequelae portend cannot be determined from these data.

Table 5 presents cell count and protein mg% found in CSF. There is no difference in mean values for either measure between the two age groups. In addition, there is no difference between those patients who are living and those who later died. The deaths were those 35 individuals who died too early to establish serologically the presence of JE virus.

We have spent little time discussing those 35 patients who died. We presume they were JE and this would give a death rate of about 24%. This is consistent with the Japanese literature, but about double that reported by Dickerson, et al, based on American troops in Korea. More recently, Ketel and Ognibene reported only one death out of 57 infections among Americans in Vietnam. This difference can probably be ascribed to the level of supportive nursing care available during the acute illness.

TABLE 1

Percent of Subjects Normal by Symptom for Each Time Zone

Symptom	Age Group	Time Zone			
		I	II	III	IV
		0-30 days	31-130 days	131-230 days	231 days or longer
Level of Consciousness	Older	37%	-	-	-
	Younger	25	-	-	-
Emotional Behavior	Older	60	72%	74%	75%
	Younger	60	68	69	71
Memory and Intellect	Older	83	66	73	74
	Younger	81	63	68	62
Convulsions	Older	49	98	100	96
	Younger	13	94	100	92
Motor Paralysis	Older	78	92	96	94
	Younger	56	75	79	75
Tremor	Older	45	45	70	80
	Younger	41	58	74	80
Ataxia	Older	59	76	93	96
	Younger	67	81	79	84
Tone	Older	41	80	98	96
	Younger	39	69	82	78
E. E. G.	Older	42	47	65	71
	Younger	18	7	21	35

TABLE 2
Psychometric Test Scores for each Time Zone¹

Psychometric Test	Time Zone			
	I 0-30 days	II 31-130 days	III 131-230 days	IV 231 or longer
Digit Span ²	30	42	46	57
Digit Symbol ²	7	22	24	33
Block Design ²	14	19	27	45
Memory Test ³	3	22	44	43

- 1 Includes those 45 subjects age eight and older.
- 2 Percentile ranking using U.S. norms.
- 3 Percent normal using Thai norms.

TABLE 3

Percent Subjects Normal and Abnormal for each Symptom in Time Zone I & IV¹

Symptoms	Age	Zone I	Zone IV	
			N%	ABN%
Convulsion	Younger	N (9)	100.0	0
		ABN (55)	69.1	7.3
	Older	N (28)	82.1	0
		ABN (27)	85.2	7.4
Involuntary Movement	Younger	N (24)	75	8.3
		ABN (32)	65.6	18.8
	Older	N (22)	81.8	9.1
		ABN (26)	69.2	23.1
Muscle Tone	Younger	N (25)	80	8
		ABN (34)	52.9	26.5
	Older	N (21)	85.7	0
		ABN (29)	86.2	6.9
Memory Test	Younger	N	-	-
		ABN (8)	87.5	12.5
	Older	N	-	-
		ABN (19)	26.3	68.4
Motor Paralysis	Younger	N (30)	76.7	10
		ABN (26)	53.9	23.1
	Older	N (41)	87.8	0
		ABN (9)	77.8	22.2
Ataxia	Younger	N (27)	85.2	3.7
		ABN (14)	78.6	14.3
	Older	N (28)	92.9	0
		ABN (18)	77.8	5.6
E. E. G.	Younger	N (8)	37.5	50
		ABN (35)	37.1	48.6
	Older	N (14)	85.7	7.1
		ABN (18)	66.7	22.2

¹ Percent values do not usually equal 100 as individuals on whom data were not available are not scored normal or abnormal. Numbers in parenthesis indicate number of subjects in each normal or abnormal group.

TABLE 4

Percent of Individuals Having Various Numbers of Abnormal Symptoms
During the First and Last Time Zones

No. of Symptoms Abnormal	Zone I		Zone IV	
	Younger	Older	Younger	Older
0	0.0%	0.0%	22.0%	23.5%
1	5.6	7.8	33.3	41.2
2	7.4	17.7	20.4	19.6
3	20.4	29.4	5.6	9.8
4	35.2	17.7	5.6	-
5	20.4	17.7	7.4	2.0
6	5.6	3.9	-	-
7	5.6	3.9	-	-
No data	-	2.0	5.6	3.9

NOTE - Includes 54 younger and 51 older cases
6 deaths are excluded, and 12 additional on which convalescent
follow-up was not possible.

TABLE 5

CSF Results

Patient Type	Cell Count per cu.mm.	Protein mg%
≤ 10 years	161+136 (n = 67)	88+64 (n = 41)
≥ 11 years	224+252 (n = 46)	97+89 (n = 39)
living	181+201 (n = 94)	86+63 (n = 64)
dead	211+152 (n = 19)	120+116 (n = 16)

In conclusion, it is apparent that while many deaths occurred, survivors recovered relatively well. Few had residuals after nine months which were severe enough to interfere with their behavioral adjustment. The present study should allow accurate prognostication of sequelae in future outbreaks of this disease in South East Asia. The consistent age differences cannot be adequately explained at this time.

7. Isolation and Identification of Methaqualone in Urine

Methaqualone (Mandrax, Parest, Quaalude, Sopor, Tuazole) abuse is a serious public health problem in the United States and abroad. Information that this drug was being abused by American teenaged children in Bangkok stimulated an attempt to develop a method for detection of this drug or its metabolites in urine. An accurate test suitable for rapid testing of large numbers of urine samples for the presence of methaqualone or its metabolites was required.

A thin layer chromatography (TLC) screening method was developed which readily detects methaqualone metabolites in urine. Urine must be subjected to hydrolysis before the metabolites can be extracted¹. Positive identification of these metabolites can then be made by gas-liquid chromatography (GLC).

Screening procedure with GLC confirmation: Urine (15 ml) is placed in a capped glass tube with concentrated HCL (5 ml) and autoclaved (110°C, 15 min, 15 psi). The hydrolyzed urine is transferred to cylindrical extraction tubes and adjusted to pH 8.5 with KOH (\approx 4 ml, 6N), then chloroform-methanol (9:1, 25 ml) is added. After agitation the aqueous layer is removed, the organic layer is dried (anhydrous sodium sulfate, 3 gms), filtered, and evaporated to dryness. The residue is spotted on TLC plates (Merck, Silica gel G). Separation is accomplished using ethylacetate-methanol-ammonium hydroxide (85:10:5) and the metabolites are located with acidified iodoplatinate reagent (0.5 g chloroplatinic acid, 9.0 g potassium iodide and 200 ml water; add equal volume 2 N HCL before use).

The metabolites (Rf 0.80, 0.75 and 0.70) from positive samples can be scraped from the TLC plate and extracted from the silica gel with methanol (3 ml). After centrifugation, the methanol is carefully transferred to narrow bottom centrifuge tubes and

evaporated to dryness. The residue is reconstituted in 20 μ l methanol and examined by GLC. The instrument used is a Varian 2700 Gas Chromatograph with hydrogen flame ionization detector using a 6 ft glass coil-shaped column containing 3% OV-1 on Gas-Chrom Q (100-120 mesh). The column temperature is 235°C using Nitrogen (25 ml/min) as the carrier gas. The retention times and Rf values of the three major metabolites are contained in Table 1.

Direct extraction of urine for GLC: Urine (15 ml) is hydrolyzed as before and extracted at pH 9.0 (sat. KOH) with chloroform-methanol (9:1, 25 ml). The organic layer is separated and extracted with H₂SO₄ (3 ml, 0.5 N). To the separated aqueous layer saturated KOH is added until pH 9.0 is reached, then chloroform-methanol (9:1, 3 ml) is added. The organic layer is separated and evaporated in narrow bottom tubes under nitrogen. The residue is reconstituted in 20 μ l methanol and examined by GLC. All three of the major metabolites can be observed with a single injection.

These procedures have been used on 650 urine samples from teenaged children in Bangkok. Of these, 2.5% were found to contain methaqualone metabolites.

Scrutiny of urine samples from the military drug urinalysis program which are positive for morphine showed that about 0.8% also contained methaqualone metabolites.

A study is now underway to determine the prevalence of methaqualone abuse in U.S. military personnel in Thailand by randomly sampling and testing urine samples received through the drug urinalysis program.

Table 1. TLC and GLC Mobility of Methaqualone Metabolites

	Rf (A)	Rf (B)	GLC*
Methaqualone	0.88	0.70	-
Codein	0.52	-	1.0
Metabolite 1	0.80	0.50	1.0
Metabolite 2	0.75	0.55	1.32
Metabolite 3	0.70	0.58	1.43

A. Ethylacetate - methanol - NH₄OH (85-10-5)

B. Ether * Retention time, relative to codeine

Project 3A062110A831 TROPICAL MEDICINE

Task 00, Tropical Medicine

Work Unit 002 Tropical and subtropical military medical research

Literature Cited.

References:

1. Burnett, D., Goudie, J.H., and Sherriff, J.M.: Detection of methaqualone and its metabolites in urine., J. Clin. Path. 22: 602-604, 1969.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION ^a	2. DATE OF SUMMARY ^a	REPORT CONTROL SYMBOL	
				DA OB 6465	72 08 01	DD-DR&E(AR)636	
3. DATE PREV SUMMARY	4. KIND OF SUMMARY	5. SUMMARY SCTY ^a	6. WORK SECURITY ^a	7. REGRADING ^a	8. DISSEM INSTN ^a	9. SPECIFIC DATA- CONTRACTOR ACCESS	10. LEVEL OF SUM
72 07 01	H. Term.	U	U	NA	NL	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	A. WORK UNIT
11. NO./CODES ^a		PROGRAM ELEMENT		PROJECT NUMBER		TASK AREA NUMBER	
a. PRIMARY		62110A		3A062110A831		00	
b. CONTRIBUTING						044	
c. OTHER WORK		CDOG 114(f)					
11. TITLE (Precede with Security Classification Code) ^a							
(U) Virus Diseases of Man and Animals							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS ^a							
002600 Biology; 003500 Clinical Medicine; 010100 Microbiology							
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
69 07		72 08		DA		C. In-House	
17. CONTRACT/GRANT				18. RESOURCES ESTIMATE		19. PROFESSIONAL MAN YRS	
a. DATES/EFFECTIVE: NA				PRECEDING			
b. NUMBER: ^a				FISCAL YEAR		6.5	
c. TYPE:				CURRENT		420	
d. KIND OF AWARD:				63		0.5	
e. CUM. AMT.						30	
20. RESPONSIBLE DOD ORGANIZATION				21. PERFORMING ORGANIZATION			
NAME: ^a Walter Reed Army Inst of Research				NAME: ^a US Army Medical Component, SEATO			
ADDRESS: ^a Washington, DC 20012				ADDRESS: ^a Bangkok, Thailand			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)			
NAME: Buescher, COL E. L.				NAME: ^a Top, LTC F. H. Jr.			
TELEPHONE: 202-576-3551				TELEPHONE: 984-4523			
				SOCIAL SECURITY ACCOUNT NUMBER: [REDACTED]			
22. GENERAL USE				ASSOCIATE INVESTIGATORS			
Foreign intelligence not considered				NAME: Edelman, LTC R.			
				NAME: Scott, MAJ R. M.			
				DA			
23. KEYWORDS (Precede EACH with Security Classification Code) ^a (U) Infectious Diseases; (U) Epidemiology; (U) Virus Ecology; (U) Arbovirus; (U) Japanese Encephalitis; (U) Rabies Virus; (U) Hepatitis							
24. TECHNICAL OBJECTIVE ^a 25. APPROACH, 26. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
23. (U) To define the ecology of viruses of military importance in Southeast Asia thus providing a rational basis for decisions which involve association with or control of that or a similar virus ecosystem.							
24. (U) Component parts of the natural viral ecosystem (e. g. vectors, hosts, reservoirs) and variables which affect these component parts (e. g. rainfall, topography, immunity) are identified and quantified through the disciplines of clinical medicine, medical entomology, epidemiology, veterinary medicine, and virology.							
25. (U) This work unit has been consolidated with work unit 002, Tropical and Subtropical Military Medical Research, Project 3A062110A831, Tropical Medicine. Progress is reported therein.							

PII Redacted

DD FORM 1498

PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 68 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION ^a	2. DATE OF SUMMARY ^a	REPORT CONTROL SYMBOL DD-DR&E(AR)636	
3. DATE PREV SUMMARY	4. KIND OF SUMMARY	5. SUMMARY SCTY ^a	6. WORK SECURITY ^a	7. REGRADING ^a	8. DOWN MSTR ^a	9. SPECIFIC DATA- CONTRACTOR ACCESS	10. LEVEL OF SUM
72 07 01	H. Term.	U	U	NA	NL	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	A. WORK UNIT
11. NO./CODES ^a		PROGRAM ELEMENT		PROJECT NUMBER		TASK AREA NUMBER	
a. PRIMARY		62110A		3A062110A831		00 045	
b. CONTRIBUTING							
c. COOPERATIVE		CDOG 114(f)					
12. TITLE (Precede with Security Classification Code) ^a							
(U) Bacterial and mycotic diseases of man and animals							
13. SCIENTIFIC AND TECHNOLOGICAL AREAS ^a							
002600 Biology; 003500 Clinical Medicine; 010100 Microbiology							
14. START DATE		15. ESTIMATED COMPLETION DATE		16. FUNDING AGENCY		17. PERFORMANCE METHOD	
69 07		CONT		DA		C. In-House	
18. CONTRACT/GRANT				19. RESOURCES ESTIMATE		20. PROFESSIONAL MAN YRS	
a. DATES/EFFECTIVE: NA				PRECEDING		b. FUNDS (in thousands)	
b. NUMBER: NA				FISCAL YEAR		72 1.3 46	
c. TYPE:				CURRENT		73 0.1 3	
d. KIND OF AWARD:				f. CUM. AMT.			
21. RESPONSIBLE DOD ORGANIZATION				22. PERFORMING ORGANIZATION			
NAME: Walter Reed Army Inst of Research				NAME: US Army Medical Component, SEATO			
ADDRESS: Washington, DC 20012				ADDRESS: Bangkok, Thailand			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)			
NAME: Buescher, E. L. COL.				NAME: McMinn, CPT M. T.			
TELEPHONE: 202-576-3551				TELEPHONE: 984-4523			
23. GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER: [REDACTED]			
Foreign intelligence not considered				ASSOCIATE INVESTIGATORS			
				NAME: Benenson, MAJ M. W.			
				NAME:			
24. KEY WORDS (Precede EACH with Security Classification Code)							
(U)Neisseria meningitidis; (U)Bacterial Diseases; (U)Mycotic Diseases; (U)Southeast Asia; (U)Diarrhea; (U)Gonorrhea; (U)Vibrio parahaemolyticus; (U) Complement							
25. TECHNICAL OBJECTIVE, 26. APPROACH, 27. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
23. (U) To identify bacterial and mycotic diseases of military importance in Southeast Asia and to provide information to aid in the diagnosis, treatment, and control of the diseases.							
24. (U) Diseases occurrence is certified by clinical and laboratory methods. Where relevant, long term surveillance of a population for occurrence of particular bacterial or mycotic diseases is instituted. Variables affecting transmission and virulence are studied in vivo and in vitro.							
25. (U) This work unit has been consolidated with work unit 002, Tropical and Sub-tropical Military Medical Research, Project 3A062110A831, Tropical Medicine. Progress is reported therein.							

PII Redacted

DD FORM 1498

PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A 1 NOV 65 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION ^a	2. DATE OF SUMMARY ^a	REPORT CONTROL SYMBOL DD-DR&E(AR)636	
3. DATE PREV SUMMARY	4. KIND OF SUMMARY	5. SUMMARY SCTY ^a	6. WORK SECURITY ^a	7. REGRADING ^a	8. DOD'S INSTN ^a	9a. SPECIFIC DATA- CONTRACTOR ACCESS	9. LEVEL OF SUM A. WORK UNIT
72 07 01	H. Term.	U	U	NA	NL	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	
10. NO./CODES: ^a	PROGRAM ELEMENT	PROJECT NUMBER		TASK AREA NUMBER	WORK UNIT NUMBER		
a. PRIMARY	62110A	3A062110A831		00	046		
b. CONTRIBUTING							
c. OTHER WORK	CDOG 114(f)						
11. TITLE (Precede with Security Classification Code) ^a (U) Parasitic Infections of Man and Animals							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS ^a 002600 Biology; 003500 Clinical Medicine; 010100 Microbiology							
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
69 07		72 08		DA		C. In-House	
17. CONTRACT/GRANT				18. RESOURCES ESTIMATE		19. PROFESSIONAL MAN YRS	
a. DATES/EFFECTIVE: NA				PRE-EXISTING		b. FUNDS (in thousands)	
b. NUMBER: ^a				FISCAL		72	
c. TYPE:				CURRENT		1.6	
d. KIND OF AWARD:				73		0.1	
19. RESPONSIBLE DOD ORGANIZATION				20. PERFORMING ORGANIZATION			
NAME: ^a Walter Reed Army Inst of Research				NAME: ^a US Army Medical Component, SEATO			
ADDRESS: ^a Washington, DC 20012				ADDRESS: ^a Bangkok, Thailand			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)			
NAME: Buescher, COL E. L.				NAME: ^a Stutz, MAJ D. R.			
TELEPHONE: 202-576-3551				TELEPHONE: 984-4523			
21. GENERAL USE ^a				SOCIAL SECURITY ACCOUNT NUMBER: [REDACTED]			
Foreign intelligence not considered				ASSOCIATE INVESTIGATORS			
				NAME:			
				NAME:			
22. KEYWORDS (Precede with Security Classification Code) ^a (U)Parasitic Diseases; (U)Malaria; (U)Host-Parasite Relationships; (U)Gnathostomiasis; (U)Immunodiagnosis; (U)Immunocnemistry; (U)Antimalarial Drugs; (U)Dirofilariasis							
23. TECHNICAL OBJECTIVE, ^a 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
23. (U) To define the ecology and basic biology of parasites of military importance in Southeast Asia providing estimates of the risk and consequences of infections with these parasites and describing effective control measures.							
24. (U) Prevalence estimates for a given parasite are made in populations of interest by serological techniques and/or by isolation and identification of the organism in clinical specimens. The disciplines of clinical medicine, veterinary medicine, medical entomology, epidemiology, and parasitology are utilized to identify life cycles and the variables which influence transmission, clinical course and chemotherapy. The disciplines of immunology and immunochemistry are utilized to clarify host-parasite relationships particularly concerning the immune response.							
25. (U) This work unit has been consolidated with work unit 002, Tropical and Sub-tropical Military Medical Research, Project 3A062110A831, Tropical Medicine. Progress is reported therein.							

DD FORM 1498
1 MAR 68

PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 65 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE.

PII Redacted

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION ^a	2. DATE OF SUMMARY ^a	REPORT CONTROL SYMBOL DD-DH&E(AR)636	
3. DATE PREV. SUMM. ^a	4. KIND OF SUMMARY	5. SUMMARY SCTY ^a	6. WORK SECURITY ^a	7. REGRADING ^a	8A. DISEASE INSTR ^a	8B. SPECIFIC DATA- CONTRACTOR ACCESS	8. LEVEL OF SUM A. WORK UNIT
72 07 01	H. Term.	U	U	NA	NL	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	
10. NO./CODES ^a	PROGRAM ELEMENT	PROJECT NUMBER		TASK AREA NUMBER		WORK UNIT NUMBER	
a. PRIMARY	62110A	3A06211A831		00		047	
b. CONTRIBUTING							
c. COORDINATING	CDOG 114(r)						
11. TITLE (Precede with Security Classification Code) ^a							
(U) Metabolic Diseases of Man and Animals							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS ^a							
003500 Clinical Medicine; 010100 Microbiology; 012900 Physiology							
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
69 07		72 08		DA		C. In-House	
17. CONTRACT/GRANT				18. RESOURCES ESTIMATE		19. PROFESSIONAL MAN YRS	
a. DATES/EFFECTIVE: NA				PRECEDING			
b. NUMBER:				FISCAL		72	
c. TYPE:				YEAR		3.1	
d. KIND OF AWARD:				CURRENT		160	
e. AMOUNT:				73		0.3	
f. CUM. AMT.						15	
20. RESPONSIBLE DOD ORGANIZATION				20. PERFORMING ORGANIZATION			
NAME: Walter Reed Army Inst of Research				NAME: US Army Medical Component, SEATO			
ADDRESS: Washington, DC 20012				ADDRESS: Bangkok, Thailand			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)			
NAME: Bueschner, COL E. L.				NAME: Davidson, LTC D. E.			
TELEPHONE: 202-576-3551				TELEPHONE: 984-4570			
21. GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER: [REDACTED]			
Foreign intelligence not considered				ASSOCIATE INVESTIGATORS			
				NAME: Rozmiarek, MAJ H.			
				NAME: Sagartz, CPT J. W. DA			
22. KEYWORDS (Precede EACH with Security Classification Code) (U) Veterinary Medicine; (U) Gibbon; (U) Leukemia; (U) Swine; (U) Insecticide; (U) Clostridium; (U) Pasteurella; (U) Diarrhea; (U) Reproduction							
23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
23. (U) To identify and characterize metabolic diseases of man and animals in Southeast Asia that may complicate or mimic infectious diseases of military importance.							
24. (U) The contribution, either wholly or in part, of metabolic disturbances resulting from malnutrition, concurrent infectious disease, or toxic agents, is evaluated in both field and laboratory studies. Clinical, biochemical, pathological, serological, and epidemiological methods are employed in this evaluation. Animal models are sought which duplicate features of these disease processes by virtue of unique physiological characteristics or susceptibility to spontaneous or induced diseases.							
25. (U) This work unit has been consolidated with work unit 002, Tropical and Sub-tropical Military Medical Research, Project 3A062110A831, Tropical Medicine. Progress is reported therein.							

^a Available to contractors upon originator's approval.

DD FORM 1498
1 MAR 68

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL	
				DA OB 6469	72 08 01	DD-DR&E(AR)436	
3. DATE PREV SUMMARY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8A. DOWN INSTRN	8B. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
72 07 01	H. Term.	U	U	NA	NL	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	A. WORK UNIT
10. NO./CODES:		PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER		WORK UNIT NUMBER	
a. PRIMARY		62110A	3A062110A831	00		048	
b. CONTRIBUTING							
c. CONTRACTING		CDOG 114(f)					
11. TITLE (Precede with Security Classification Code)							
(U) Rickettsial Diseases of Man and Animals							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS							
002600 Biology; 003500 Clinical Medicine; 010100 Microbiology							
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
69 07		72 08		DA		C. In-House	
17. CONTRACT/GRANT				18. RESOURCES ESTIMATE		19. PROFESSIONAL MAN YRS	
a. DATES/EFFECTIVE: NA				PRECEDING		b. FUNDS (in thousands)	
b. NUMBER:				FISCAL YEAR		c. FUNDS (in thousands)	
c. TYPE:				CURRENT		d. FUNDS (in thousands)	
e. KIND OF AWARD:				73		1	
19. RESPONSIBLE DOD ORGANIZATION				20. PERFORMING ORGANIZATION			
NAME: Walter Reed Army Inst of Research				NAME: US Army Medical Component, SEATO			
ADDRESS: Washington, DC 20012				ADDRESS: Bangkok, Thailand			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)			
NAME: Buescher, COL E. I.				NAME: Winter, LTC P. E.			
TELEPHONE: 202-576-3531				TELEPHONE: 884-4523			
21. GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER: [REDACTED]			
Foreign intelligence not considered				ASSOCIATE INVESTIGATORS			
				NAME:			
				NAME: DA			
22. KEYWORDS (Precede EACH with Security Classification Code)							
(U) Rickettsial Diseases; (U) Scrub Typhus; (U) Murine Typhus; (U) Leptothrombidium arenicola							
23. TECHNICAL OBJECTIVE. 24. APPROACH. 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
23. (U) To define the biology of rickettsial diseases of military importance in Thailand.							
24. (U) Disease occurrence in Thailand is determined by case detection and laboratory methods. The disciplines of clinical medicine, medical entomology, epidemiology, and rickettsiology are used to identify the various components of the ecosystem (e. g. vectors, hosts, reservoirs).							
25. (U) This work unit has been consolidated with work unit 002, Tropical and Sub-tropical Military Medical Research, Project 3A062110A831, Tropical Medicine. Progress is reported therein.							

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DD FORM 1 MAR 68 1498

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION ^a	2. DATE OF SUMMARY ^a	REPORT CONTROL SYMBOL DD-DR&E(AR)636	
3. DATE PREVIOUS ^a	4. KIND OF SUMMARY	5. SUMMARY SCY ^a	6. WORK SECURITY ^a	7. REGRADING ^a	8. ORIGIN INST ^a	9. SPECIFIC DATA - CONTRACTOR / CCEB ^a	10. LEVEL OF SUM A. WORK UNIT
72 07 01	H. Term.	U	U	NA	NL	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	
11. NO./CODES ^a		12. PROGRAM ELEMENT		13. PROJECT NUMBER		14. TASK AREA NUMBER	
A. PRIMARY		62110A		3A062110A831		00	
B. CONTRIBUTING						109	
C. CONTRIBUTING		CDOG 114(f)					
11. TITLE (Precede with Security Classification Code) ^a							
(U) Psychiatry and Behavioral Studies							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS ^a							
013400 Psychology; 003500 Clinical Medicine							
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
69 07		CONT		DA		C. In-House	
17. CONTRACT/GRANT				18. RESOURCES ESTIMATE		19. PROFESSIONAL MAN YRS	
A. DATES/EFFECTIVE: NA				PRECEDING		B. FUNDS (in thousands)	
B. NUMBER:				72		1.5	
C. TYPE:				FISCAL YEAR		110	
D. KIND OF AWARD:				CURRENT		0.1	
E. CUM. AMT.				73		10	
20. RESPONSIBLE DOD ORGANIZATION				21. PERFORMING ORGANIZATION			
NAME: Walter Reed Army Inst of Research				NAME: US Army Medical Component, SEATO			
ADDRESS: Washington, DC 20012				ADDRESS: Bangkok, Thailand			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)			
NAME: Buescher, COL E. L.				NAME: Schneider, CPT R. J.			
TELEPHONE: 202-576-3551				TELEPHONE: 984-4544			
22. GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER: [REDACTED]			
Foreign intelligence not considered				ASSOCIATE INVESTIGATORS			
				NAME: Noll, MAJ W. W.			
				NAME: Jewell, CPT J. S.			
23. KEYWORDS (Precede EACH with Security Classification Code) (U) Psychiatry; (U) Human Behavior; (U) Neurological Diseases; (U) Human Volunteer; (U) Drug Abuse							
24. TECHNICAL OBJECTIVE, 25. APPROACH, 26. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
23. (U) To study variables in the host environment that may adversely affect the performance of duty, principally through alterations in human behavior, and the development of biochemical tests to determine the presence of certain drugs in urine.							
24. (U) American and Thai professionals in the mental health area, working with trained technicians utilizing systematic observation of human behavior, observe the impact of such agents as Japanese encephalitis and indiscriminate drug usage upon the immediate and long-term performance of individuals in natural or alien environments							
25. (U) This work unit has been consolidated with work unit 002, Tropical and Sub-tropical Military Medical Research, Project 3A062110A831, Tropical Medicine. Progress is reported therein.							

^a Available to contractors upon originator's approval.

DD FORM 1498

PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A 1 NOV 65 AND 1498-1 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE

PROJECT 3A062110A821
COMBAT SURGERY

Task 00
Combat Surgery

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION ^a	2. DATE OF SUMMARY ^a	REPORT CONTROL SYMBOL	
				DA OA 6466	73 07 01	DD-DR&E(AR)636	
3. DATE PREV SUMMARY	4. KIND OF SUMMARY	5. SUMMARY SCTY ^a	6. WORK SECURITY ^a	7. REGRADING ^a	8. DISEM INSTR ^a	9. SPECIFIC DATA - CONTRACTOR ACCESS	10. LEVEL OF SUM
72 07 01	D Change	U	U	NA	NL	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	A. WORK UNIT
10. NO./CODES ^a		PROGRAM ELEMENT		PROJECT NUMBER		TASK AREA NUMBER	
		62110A		3A062110A821		00	
11. PRIMARY						120	
12. CONTRIBUTING							
13. CONTRIBUTING		CDOG 114(F)					
11. TITLE (Precede with Security Classification Code) ^a							
(U) Wound healing							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS ^a							
003500 Clinical Medicine 012900 Physiology							
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
54 09		CONT		DA		C In-House	
17. CONTRACT/GRANT NA				18. RESOURCES ESTIMATE		19. PROFESSIONAL MAN YRS	
a. DATES/EFFECTIVE:				PREVIOUS		b. FUNDS (in thousands)	
b. NUMBER:				73		3.0	
c. TYPE:				FISCAL YEAR		150	
d. KIND OF AWARD:				74		3.0	
e. CUM. AMT.						150	
20. RESPONSIBLE DOD ORGANIZATION				21. PERFORMING ORGANIZATION			
NAME: Walter Reed Army Institute of Research				NAME: Walter Reed Army Institute of Research			
ADDRESS: Washington, D. C. 20012				Division of Surgery			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)			
NAME: Buescher, COL T. L.				NAME: Spees, COL E. K.			
TELEPHONE: 202 576 - 3551				TELEPHONE: 202 576 - 3250			
22. GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER: [REDACTED]			
Foreign Intelligence Not Considered				ASSOCIATE INVESTIGATORS			
				NAME: Burleson, MAJ R. L.			
				NAME: Deshon, MAJ G. E., Jr.			
				DA			
23. KEYWORDS (Precede EACH with Security Classification Code) (U) Immune Mechanisms; (U) Rejections; (U) Abscess; (U) Combat Injury							
24. TECHNICAL OBJECTIVE, 25. APPROACH, 26. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
23 (U) Better understanding of the immune mechanisms involved in allografts and foreign body rejection; tissue matching and cell cytotoxicity measurements to predict allograft success; antigen modification; detection of abscesses scintigraphically; defining the pathophysiology of tissue infection; delineation of steroid and uremic effects on leukocyte function.							
24 (U) The parameters of sensitization, rejection, tissue matching and antigen modification are being investigated through evaluation of vein allografts, one way mixed lymphocyte culture, chromium release cytotoxicity assays and antigen modification with liver extracts. Abscess scintiscanning is being evaluated. 131 I labeled staph in phagocytic and bacterial kill assays is being utilized to study the dynamics of leukocyte function in infected tissue, the effects of steroids and uremia on leukocyte function.							
25 (U) 72 07 - 73 06. (a) The pathology of vein graft rejection in regards to host endothelialization has been defined. (b) One way MLC and 51 Cr release cytotoxicity assays are showing predictive value in the transplant program. (c) Prevention of sensitizing ability of intact spleen cells by hepatic extract pretreatment confirmed by mouse skin grafts. (MAJ Deshon received Hoff Medal for this work.) (d) Scintigraphic detection of abscesses accomplished with labeled leukocytes. (e) The production of an antiphagocytic substance by leukocytes in rabbit studies indicates a role in the perpetuation of tissue infection. The study is continuing. (f) Rabbit and dog studies indicate no effect of high dose steroid therapy on the phagocytic or intracellular kill capability of peripheral blood phagocytes. (g) Studies indicate dialysis corrects marked phagocytic functional impairment.							
For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 72-30 Jun 73.							

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DD FORM 1498

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Project 3A062110A821 COMBAT SURGERY

Task 00 Combat Surgery

Work Unit 120 Wound healing

Investigators.

Principal: COL Everett K. Spees, MC

Associate: MAJ Richard L. Burleson, MC; MAJ George E. Deshon, Jr., MC*

Better understanding of the immune mechanisms involved in the rejection of allografts and foreign bodies; tissue matching and cell cytotoxicity predictive measurements to predict allograft success; prevention of sensitization by antigen modification; detection of abscesses scintigraphically; defining the pathophysiology of tissue infection; delineation of the effect of steroids on leukocyte function; delineation of the effect of uremia on leukocyte function.

The parameters of sensitization, rejection, tissue matching and antigen modification are being investigated through evaluation of vein allografts, one way mixed lymphocyte culture, chromium release cytotoxicity assays and antigen modification with liver extracts. Abscess scintiscanning is being evaluated with ^{67}Ga labeled autologous leukocytes and intravenous ^{67}Ga citrate. Quantitative phagocytic and bacterial kill assays employing ^{131}I -labeled staph are being utilized to study the dynamics of leukocyte function in infected tissue, the effects of steroid on leukocyte function, and the effect of uremia on leukocyte function.

The pathology of vein graft rejection in regards to host endothelialization has been defined. Utilization of the one way MLC and ^{51}Cr release cytotoxicity assays are showing predictive value in the clinical renal transplant program. Sensitization by acellular splenic antigen and living spleen cells has been abrogated by liver extracts as monitored in a mouse skin graft system (work by MAJ Deshon received the Hoff award). Scintigraphic detection of abscesses with ^{67}Ga labeled autologous leukocytes has been accomplished and the technique is now being evaluated in human patients. Phagocytic studies in rabbits indicate the production of an antiphagocytic substance by leukocytes that may play a role in the perpetuation of tissue infection - confirmation and purification studies are in progress. Chronic studies in rabbits and dogs indicate no effect of high dose steroid therapy on the phagocytic or intracellular kill capability of peripheral blood phagocytes - final studies are in progress. Preliminary studies in humans indicate a marked leukocyte phagocytic functional impairment in uremia corrected by dialysis.

*Fellow, Research Training Fellowship Program, WRAIR, 1972-1973

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION ^a	2. DATE OF SUMMARY ^a	REPORT CONTROL SYMBOL	
				DA QA 6467	73 07 01	DD-DR&E(AR)836	
3. DATE PREV SUMRY	4. KIND OF SUMMARY	5. SUMMARY SCTY ^a	6. WORK SECURITY ^a	7. REGRADING ^a	8A. DR&E INSTR ^a	8B. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
72 07 01	D Change	U	U	NA	NL	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	A. WORK UNIT
10. NO./CODES ^a	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER		WORK UNIT NUMBER		
A. PRIMARY	62110A	3A062110A821	00		121		
B. CONTRIBUTING							
C. CONTRIBUTING	CDOG 114(F)						
11. TITLE (Precede with Security Classification Code) ^a							
(U) Responses to trauma							
12. SCIENTIFIC AND TECHNOLOGICAL AREA ^a							
008800 Life Support 016200 Stress Physiology							
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
63 09		CONT		DA		C In-House	
17. CONTRACT/GRANT NA				18. RESOURCES ESTIMATE		19. PROFESSIONAL MAN YRS	
A. DATES/EFFECTIVE:				PRECEDING		FUND\$ (In thousands)	
B. NUMBER ^a				73		4.5	
C. TYPE:				FISCAL YEAR		125	
D. KIND OF AWARD:				74		4.5	
E. CUM. AMT.						125	
19. RESPONSIBLE DOD ORGANIZATION				20. PERFORMING ORGANIZATION			
NAME ^a Walter Reed Army Institute of Research				NAME ^a Walter Reed Army Institute of Research			
ADDRESS ^a Washington, D. C. 20012				ADDRESS ^a Washington, D. C. 20012			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)			
NAME: Buescher, COL E. L.				NAME ^a Swan, LTC K. G.			
TELEPHONE: 202 576 - 3551				TELEPHONE: 202 576 - 3791			
21. GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER: [REDACTED]			
Foreign Intelligence Not Considered				ASSOCIATE INVESTIGATORS			
				NAME: Reynolds, LTC D. G.			
				NAME: Ritchie, LTC W. P.			
				DA			
22. KEYWORDS (Precede EACH with Security Classification Code)							
(U) Stress Ulcer; (U) Shock; (U) Ulcerogenesis;							
(U) Ion Transport; (U) Splanchnic Hemodynamics;							
23. TECHNICAL OBJECTIVE ^a 24. APPROACH. 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with security Classification Code.)							
23 (U) Elucidate the pathogenesis of irreversible shock. Study the pathophysiology of stress ulcer, a complication that causes massive gastrointestinal bleeding in 3% of severely wounded with a 40% mortality.							
24 (U) Study mesenteric hemodynamics in endotoxin shock with emphasis on species variation. Search for a better model for stress ulcer. Study ion transport across the gastric mucosa and the effects of metabolic inhibitors and hemorrhage on gastric mucosal permeability.							
25 (U) 72 07 - 73 06. A lethal dose of endotoxin given intravenously caused profound hypotension in baboons but no change in mesenteric, hepatic, or renal arterial blood flow. The canine response differs in that each of these vascular beds become ischemic in response to equivalent treatment. The silicone rubber injection technique has been used to define a specific intestinal vascular lesion in the dog but is absent in the monkey. The data suggest that the splanchnic viscera are not primary sites of pathology during shock in monkeys and presumably man. In vivo and in vitro studies on mammalian stomach electrical and pH gradients demonstrated the gastric mucosal barrier (GMB) to be impaired by inhibition of ATP synthesis, anaerobic glycolysis and hemorrhage. Bile salts and acetylsalicylic acid damage the GMB by altering membrane electrical characteristics. These effects may be related to the pathogenesis of gastric stress ulcers. Restraint stressed rats that were provided 5.0 gm % of glucose to drink developed fewer stress erosions than did control rats. Glucose did not protect at lower concentrations.							
For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 72-30 Jun 73.							
^a Available to contractors upon originator's approval							

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DD FORM 1498

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Project 3A062110A821 COMBAT SURGERY

Task 00 Combat Surgery

Work Unit 121 Responses to trauma

Investigators.

Principal: LTC Kenneth G. Swan, MC

Associate: MAJ Creighton E Wright, MC; LTC Robert W. Hobson, MC;
LTC David G. Reynolds, MSC; LTC John F. Mullane, MC;
James T. Sayre; MAJ Peter F. Casterline; SP6 Ernest
C. Alix; MAJ Michael D. Sulkin, MC; MAJ Alfred S. Gervin, MC

1. Peripheral Arterial Flow and Venous Occlusion.

a. Background: Arteriovenous trauma in the lower extremity has usually been managed by arterial reconstruction and venous ligation of the injured concomitant vein. Clinical series from Vietnam have documented the occurrence of gangrene due to acute venous insufficiency. The hemodynamics of acute venous occlusion have not been clearly defined. The present studies were designed to evaluate the hemodynamic responses to acute and chronic venous occlusion in the hindlimb and in addition were designed to evaluate means whereby adverse hemodynamic sequela might be elevated.

2. Autoregulatory Escape in Hepatic Circulation of Subhuman Primates.

Adrenergic mechanisms of the splanchnic viscera have been evaluated in different vascular beds in several species of animals. The presence and absence of β adrenergic receptors in the liver of the primate and the primate responses to catecholamine infusions has not previously been documented. The presence of autoregulation differs in the dog and cat models previously examined. Pharmacologic manipulation of the splanchnic visceral blood flow with particular reference to the control of hemorrhage is based upon the responses of vascular beds to constant infusion of pharmacological agents. The effort is to examine the presence of α and β adrenergic receptors and also the presence of autoregulatory escape in the hepatic circulation of the subhuman primate, the anubis baboon.

3. Pathophysiology of Intraarterial Injection of Drugs.

The inadvertent intraarterial injection of drugs of abuse including amphetamines, barbiturates, analgesics, and narcotics has on occasion been followed by significant peripheral vascular insufficiency with subsequent gangrene. The etiologic mechanism for this syndrome has previously been postulated to be acute vasospasm induced by the drugs. This hypothesis was examined in the canine hindlimb

with the finding that dextroamphetamine and methamphetamine are significant vasoconstrictors however Darvon, thiopental, secobarbital sodium, pentobarbital sodium, morphine, methadone, and heroin are vasodilators when injected intraarterially. Additional mechanisms for the etiology of the vascular insufficiency syndrome must be found.

4. Peripheral Arterial Flow in Venous Occlusion Before and After a Lumbar Sympathectomy in the Baboon.

The ligation of injured veins in the situation of combined arterial and venous injuries has been the common management. Recent clinical series have demonstrated significant complications from the ligation of extremity veins. Previous work in this laboratory on the canine model has demonstrated the hemodynamic sequelae which follow femoral venous ligation. Since the primate model is presumably similar to man with particular regard to the collateral venous circulation, the problem of femoral venous ligation has been investigated in the baboon. Femoral venous ligation reduces femoral arterial flow while increasing femoral venous pressure and peripheral resistance in the hindlimb. The addition of lumbar sympathectomy and presumably lumbar sympathetic blockade significantly improves the arterial flow with no significant deleterious effects on peripheral venous pressure. The recommendation is that, in the clinical situation when major peripheral venous ligation is unavoidable, lumbar sympathectomy should be considered as a useful adjuvant technique.

5. Study of Factors Present After Trauma.

Experiments completed this year have been designed to study factors present after trauma that might increase susceptibility to 1) stress ulcer formation or 2) pulmonary infection.

The effects of starvation, graded glucose ingestion, gastric congestion and oxygen toxicity on stress ulcer formation have been studied. Seven hours of wire mesh restraint was used as a stress model for the rat. Rats were starved for 4 days or given 1.0, 2.5 or 5.0% glucose as a drinking solution. The latter concentration of glucose prevented the increased incidence of gastric stress lesions with starvation. This was not related to changes in gastric acid production, oxygen consumption or gastric absorption of ingested glucose. A critical degree of caloric intake appeared to be necessary to protect the stomach from stress.

When rats were studied 2, 4 and 7 days after portal vein constriction, there was an increased incidence of stress induced gastric lesions. At 14 days when the portal pressure had returned to normal, the rat's stomach was no longer susceptible to stress. Vagotomy protected the congested stomach from the effects of stress.

Rats that breathed 100% oxygen for 48 hours had an increased incidence of stress induced gastric lesions. These rats did not gain as much weight as controls and had a lower mean arterial pressure. After 48 hours of 100% O₂, arterial pH and pCO₂ were normal but arterial pO₂ was 280 mm Hg instead of 400 mm Hg. Portal pressure remained normal despite the pulmonary edema with 100% O₂ and gastric congestion was not of etiological significance.

The effects of acute blood loss, dehydration, heart failure, aspiration of blood and stress on intrapulmonary antibacterial defenses of the rat have been studied. Bacteriological portions of the study were performed at Channing Laboratory, Boston and the physiological and biochemical portions were performed at WRAIR. The effect of these five variables on intrapulmonary bacterial inactivation, alveolar bubble stability ratio, arterial pH, pCO₂ and pO₂, numbers of viable macrophages recovered by bronchial lavage in vitro, oxygen consumption of alveolar macrophages and acid phosphate β glucuronidase and leucine aminopeptidase content of the macrophages were studied. Intrapulmonary bacterial inactivation was depressed in all five models. This was accompanied by decreased alveolar surface activity in all five studies. Major pulmonary morphological changes were present with aspiration of blood and heart failure. Hypoxia was present for only a few hours after blood loss. The viability, oxygen consumption and enzyme content of macrophages recovered by bronchial lavage were similar to controls in all five groups.

Recommendations for further study would include the effect of intravenous nutrition on stress ulcer formation and changes in the immunobiology of the alveolar macrophage in the various trauma related conditions.

6. Two projects have been pursued: (a) Ex vivo perfusion of the canine stomach and (b) fibrinolytic activity in the pseudo-intima of Dacron aortic grafts.

In developing a system of ex vivo perfusion of the stomach several problems have been encountered of which one has to date defied solution. This involves the development of edema of the mucosa. Work is continuing to solve this problem.

The study of appearance of fibrinolytic activator in healing Dacron grafts has been most rewarding. A method of accurately measuring fibrinolytic activator has been developed for our laboratory. Several innovations in technique have been devised and a methods manuscript is in progress. Appearance of fibrinolytic activator has been shown to be present at least as early as one day after graft insertion and correlates well with the appearance of pseudo-intima. Completion of work on this data should be available by the end of August for preparation of a manuscript.

Several other projects involving fibrinolytic activator in intima of rejecting vessels, pericardium, and vessels exposed to infused intravenous fluids and other medications are underway.

Project 3A062110A821 COMBAT SURGERY

Task 00 Combat Surgery

Work Unit 121 Responses to trauma

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION ^a	2. DATE OF SUMMARY ^a	REPORT CONTROL SYMBOL	
				DA OA 6468	72 08 01	DD-DR&E(AR)636	
3. DATE PREV SUMMARY	4. KIND OF SUMMARY	5. SUMMARY SCTY ^a	6. WORK SECURITY ^a	7. REGRADING ^a	8A. DR&E INSTR ^a	8B. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
72 07 01	H. Termination	U	U	NA	NL	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	A. WORK UNIT
10. NO / CODES ^a		PROGRAM ELEMENT		PROJECT NUMBER		TASK AREA NUMBER	
		62110A		3A062110A821		00	
11. PRIMARY						122	
12. CONTRIBUTING							
13. CONTRIBUTING		CDOG 114(f)					
14. TITLE (Precede with Security Classification Code) ^a							
Anesthesia and pulmonary complications of combat injury							
15. SCIENTIFIC AND TECHNOLOGICAL AREAS ^a							
002400 Bioengineering 012900 Physiology 008800 Life Support							
16. START DATE		17. ESTIMATED COMPLETION DATE		18. FUNDING AGENCY		19. PERFORMANCE METHOD	
58 05		72 08		DA		C. In-House	
20. CONTRACT/GRANT NA				21. RESOURCES ESTIMATE		22. PROFESSIONAL MAN YRS	
A. DATES/EFFECTIVE:				B. PRECEDING		C. FUNDS (in thousands)	
B. NUMBER:				72		4	
C. TYPE:				73		125	
D. KIND OF AWARD:				FISCAL YEAR			
E. CUM. AMT.							
23. RESPONSIBLE DOD ORGANIZATION				24. PERFORMING ORGANIZATION			
NAME: Walter Reed Army Institute of Research				NAME: Walter Reed Army Institute of Research			
ADDRESS: Washington, D. C. 20012				ADDRESS: Washington, D. C. 20012			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)			
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TELEPHONE: 202 576 - 3551				TELEPHONE: 202 576 - 2007			
25. GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER: [REDACTED]			
Foreign Intelligence Not Considered				ASSOCIATE INVESTIGATORS			
				NAME:			
				DA			
26. KEYWORDS (Precede EACH with Security Classification Code) (U) Intrapulmonary Antibacterial Defense Mechanisms; (U) Acute Blood Loss; (U) Dehydration; (U) Heart Failure; (U) Aspiration of Blood; (U) Hypoxia							
27. TECHNICAL OBJECTIVE, 28. APPROACH, 29. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
23 (U) The effects of acute blood loss, dehydration, heart failure, aspiration of blood, and stress on intrapulmonary antibacterial defenses of the rat have been measured to evaluate the effects of trauma on the susceptibility of the injured patient to pulmonary infection.							
24 (U) Intrapulmonary bacterial inactivation and alveolar surfactant activity were depressed in each group. Extensive morphologic alterations in the lungs followed experimental aspiration of blood and experimental heart failure in rats. Hypoxia was associated with acute blood loss for only a few hours following experimental hemorrhage. The viability, oxygen consumption, and enzyme content of macrophages recovered by bronchial lavage were similar to controls in all five groups.							
25 (U) Termination of this work unit is due to combination with another work unit, 3A062110A821 00 121.							

^aAvailable to contractor, upon originator's approval.

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PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 66 AND 1498-1, 1 MAR 66 (FOR ARMY USE) ARE OBSOLETE.

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PROJECT 3A062110A822
MILITARY INTERNAL MEDICINE

Task 00
Military Internal Medicine

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION ^a	2. DATE OF SUMMARY ^a	REPORT CONTROL SYMBOL	
				DA OA 6469	73 07 01	DD-DR&E(AK)636	
3. DATE PREV. WRTY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING ^a	8. ONE'S INSTR ^a	9a. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
72 07 01	D. Change	U	U	NA	NL	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	A. WORK UNIT
10. NO. / CODES ^a	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER			
A. PRIMARY	62110A	3A062110A822	00	120			
B. CONTRIBUTING							
XXXXXXX	DOG 114(F)						
11. TITLE (Precede with Security Classification Code) ^a							
(U) Metabolic Response to Disease and Injury							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS ^a							
012900 PHYSIOLOGY		003500 CLINICAL MEDICINE		002300 BIOCHEMISTRY			
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
63 08		CONT		DA		C. In-House	
17. CONTRACT GRANT				18. RESOURCES ESTIMATE		A. PROFESSIONAL MAN YRS	
A. DATES/EFFECTIVE: NA				PRECEDENCE		B. FUNDS (in thousands)	
B. NUMBER ^a				FISCAL YEAR		73	
C. TYPE				CURRENCY		20	
D. AMOUNT:						365	
E. KIND OF AWARD:				74		20	
F. CUM. AMT.						365	
19. RESPONSIBLE DOD ORGANIZATION				20. PERFORMING ORGANIZATION			
NAME: Walter Reed Army Institute of Research				NAME: Walter Reed Army Institute of Research			
ADDRESS: Washington, DC 20012				Division of Medicine			
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RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)			
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				SOCIAL SECURITY ACCOUNT NUMBER: [REDACTED]			
21. GENERAL USE				ASSOCIATE INVESTIGATORS			
Foreign Intelligence Not Considered				NAME: Wartofsky, LTC, L.			
				NAME: Schaaf, M.D., M.			
				DA			
22. KEYWORDS (Precede EACH with Security Classification Code)							
(U) Metabolic; (U) Stress; (U) Endocrine; (U) Hormone							
23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Precede individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
23. (U) Investigation into basic mechanisms of diseases of military importance and the metabolic response of patients during stress of disease and injury to provide rational approach to therapy.							
24. (U) Metabolic balance studies with precise collection of biologic samples from patients under rigidly controlled diet, drugs, and activity. Development of techniques to measure alterations in homeostasis produced by disease or drugs. Provide clinical support and teaching for the Walter Reed Army Medical Center.							
25. (U) 72 07 - 73 06. Investigations of alterations in endocrine hormones and their relationships to changes in body metabolism which occur during trauma, stress, immobilization, and infection were conducted. Thyroid gland function is suppressed during acute malaria infection. Thyroid stimulating hormone and prolactin were transiently decreased during the first to third day of the infection. Prolactin, a hormone which increases with many stresses, did not increase, and responses of both hormones to intravenous thyrotropic stimulating hormone releasing factor was normal during malaria infection. Urinary cyclic three prime five prime adenosine monophosphate is suppressed by calcium infusion in both idiopathic hypercalciuria and adenomatous hyperparathyroidism, confirming the non-autonomous nature of the latter. Thirteen acromegalic patients were treated via transsphenoidal microsurgery with excellent response. Selective venous catheterization and measurement of parathyroid hormone has proved to be useful in localizing adenomas and predicting hyperplasia in fifteen patients. For technical reports see Walter Reed Army Institute of Research Annual Progress Report, 1 July 72 - 30 June 73.							

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Project 3A062110A822 MILITARY INTERNAL MEDICINE

Task 00, Military Internal Medicine

Work Unit 120, Metabolic response to disease and injury

Investigators:

Principal: COL Jerry M. Earll, MC

Associates: Marcus Schaaf, M.D.; LTC Leonard Wartofsky, MC;
MAJ Richard C. Dimond, MC; MAJ Gordon L. Noel, MC;
MAJ Jack M. Monchik, MC; Joseph Bruton, Ph. D.

Description

This work unit is concerned with investigations into basic mechanisms of diseases of military importance and the metabolic responses occurring during stress of disease and injury to provide rational approaches to therapy. Metabolic balance studies are utilized with precise collections of biologic samples from patients during rigid control of diet, drugs, and activity. In addition, support is afforded Walter Reed Army Medical Center in training house staff, four endocrine fellows, diagnosis and treatment of endocrine patients, and technical laboratory support to other departments. The unit maintains the capability of mounting field studies.

Progress

1. Thyroid Metabolism.

Studies in four humans infected with malaria have revealed that stimulation of thyroid stimulating hormone (TSH) with repetitive doses of thyrotropin releasing factor (TRF) exhausts the pituitary TSH response. TSH levels responded to TRH stimulation during malaria infection. Prolactin, which seems to respond even better to TRF than TSH, continues to respond with repeated injections. Prolactin which responds to mental stress and exhaustive exercise, fails to respond to the fever and stress associated with malaria.

The release of thyroid stimulating hormones (TSH) and prolactin by bolus injections of large doses of TRH has been widely studied. Considerable doubt remains as to whether TRH is more effective in releasing prolactin than TSH, what the smallest effective dose of TRH is in releasing the two hormones, and whether the pituitary responds to TRH in an "all-or-none" fashion. To answer these questions we have given small doses of TRH in constant infusions via Harvard pump to ten normal volunteers, five males and five females. Blood was drawn at 15 minute intervals for 450 minutes while TRH was given at the following rates: 25 ng/min,

75 ng/min, 250 ng/min each for 120 minutes, and 750 ng/min for 90 minutes, after which a 500 mcg bolus of TRH was administered. In both males and females TSH infusion at 250 ng/min (HPr $p < 0.005$, TSH $p < 0.025$). TSH and HPr values plateaued during the final 60 minutes at this infusion rate, but rose again during the first 30 to 60 minutes of the next infusion rate (750 ng/min) and then plateaued once more. After the 500 mcg bolus of TRH, TSH and HPr rose further in all individuals. Prolactin values were significantly higher in females than in males at all times; TSH levels were significantly higher in females during the 250 and 750 ng/min infusions and after the TRH bolus.

By employing a technique of constant TRH infusion, we have demonstrated (1) the threshold dose of TRH is greater than 75 ng/min and less than or equal to 250 ng/min for both TSH and HPr; (2) TSH and HPr responses are parallel at each infusion rate; (3) although the smallest effective dose of TRH is the same in males and females, females have a greater increase of both TSH and HPr; (4) at these very small doses there is a clear dose-response relationship of both TSH and HPr to TRH.

One hundred and seventy-five studies of TSH response to TRH have been completed and TRH is definitely a useful agent for the assessment of disorders of the hypothalamic-pituitary-thyroid axis. TRH significantly stimulated growth hormone in 25% of 12 acromegalic patients.

The thyrochron, a self-contained direct digital readout Achilles reflex time, has proved to be highly accurate and easy to operate. Mild exercise such as walking, speeds the reflex (52 ± 22 msec) and cold slows it (101 ± 38 msec) so that these conditions must be considered and controlled to improve the usefulness of the Achilles reflex time.

2. Calcium Metabolism.

Seventeen patients with primary hyperparathyroidism have undergone selective venous catheterization. Fifteen of these patients have never previously had surgery. Parathyroid adenoma was correctly predicted and lateralized in 12 of these 15 patients who have never previously had surgery. The remaining three patients had hyperplasia correctly predicted pre-operatively. Two patients who had previous surgery have had selective venous catheterization. In one patient hyperplasia was predicted and an adenoma was found. The other patient is awaiting surgery. It is concluded that selective venous catheterization has proven to be a valuable means of lateralizing parathyroid adenoma and in pre-operatively predicting hyperplasia or adenoma in patients who have never previously had surgery. We presently do not have enough data on patients who have had previous surgery.

These plasma samples were obtained from normal individuals and endocrine patients. Results from each of these procedures were inter-compared. The fluorometric procedure and the competitive protein-binding assay compared favorably with results obtained from the more elaborate and difficult double-isotope derivative method. The Porter-Silber method showed a lack of specificity.

4. Polypeptide Hormone Metabolism.

Application of modern technology to an old surgical approach has led to a treatment of acromegaly that promises improved therapy with eradication of the tumor and preservation of normal pituitary function. Using a dissecting microscope and a transsphenoidal approach to the sella turcica, it is possible to distinguish normal pituitary from tumor under direct vision and in most cases preserve the normal gland. We have treated 13 active acromegalics. Two patients had hypopituitarism before treatment which was not improved by surgery; two patients with normal pituitary function pre-operatively pituitary evaluation but are doing well off replacement medication. The other six patients have normal anterior pituitary function. Transient diabetes insipidus was noted in the immediate post-operative period in almost all patients. One patient requires vasopressin in oil two months after surgery. One patient developed transient aphasia and hemiparesis six days after surgery, and one was blinded in one eye from an orbital fracture. This complication should not recur due to changes in techniques. Pre-operative basal growth hormone (GH) levels ranged from 9 to 98 ng/ml. Follow-up GH data ranging from two weeks to one year is available in 10 patients. Basal GH was undetectable in five patients, < 3 ng/ml in one; < 6 ng/ml in two, < 10 ng/ml in one, and in one patient with prior unsuccessful yttrium implants the GH dropped from 98 to 39 ng/ml. Twelve of 13 patients noted decrease in ring size or other soft tissue changes within three to five days after surgery. Transsphenoidal microsurgery for acromegaly may become the treatment of choice since it offers several distinct advantages: (1) it is a relatively simple neurosurgical procedure; (2) post-operative recovery is rapid; (3) growth hormone is rapidly lowered to levels which allow dramatic clinical response; and (4) surgery under direct observation with the microscope allows removal of abnormal tissue and selective preservation of normal tissue and pituitary function in most of the cases.

Stimulation and suppression of plasma prolactin in patients with pituitary disease has been carried out in nine patients with various types of galactorrhea. All patients had extensive standard pituitary function tests prior to the prolactin studies. TRH was found to release prolactin from patients with non-pituitary tumor-related galactorrhea; chlorpromazine released prolactin in patients with intact hypothalamic-pituitary connections. Breast stimulation was not an effective stimulus

to prolactin release in any individual. Too few patients were studied during sleep to draw any conclusions at this time. In over 20 patients studied with pituitary tumors but without galactorrhea, prolactin release has been preserved more frequently than any other pituitary hormone; about 20% have elevated prolactin levels without evidence of hormonal excess. It can be concluded that prolactin response to specific stimulatory and suppressive tests is useful in determining the etiology of galactorrhea. Prolactin response is also an excellent parameter of pituitary function and preservation in patients with pituitary disease.

It is well established that several of the polypeptide hormones may exist in the circulation in heterogeneous forms. To explore the possibility that a similar phenomenon may occur among glycoprotein hormones, we have recently compared purified pituitary thyrotropin (hTSH) to serum hTSH by gel chromatography. Standard pituitary hTSH (Mill Hill-A in hypopituitary serum) and serum samples from three patients with primary hypothyroidism, two of whom were also studied after thyrotropin releasing factor (TRF) administration, were examined. Each sample was co-chromatographed with ^{125}I -hTSH (National Pituitary Agency, NIAMDD) serving as an internal standard and with ^{125}I -bovine thyroglobulin and ^{125}I to establish the void volume and total volume. One ml samples were applied to, and 1 ml fractions were collected from, a 1.5 x 80 cm column of Sephadex G-100, operated at a flow rate of 3.4 ml/hr at 4° C. In each fraction, labeled pituitary TSH was measured in terms of radioactivity and unlabeled pituitary TSH was symmetric in all experiments and was indistinguishable from unlabeled pituitary TSH, validating the labeled internal standard as a chromatographic marker for pituitary TSH; the K_{av} for pituitary TSH was 0.326 ± 0.002 S.D. However, the elution chromatogram of unlabeled serum TSH for each of the five samples was clearly distinguished from the pituitary TSH marker by its asymmetry and by its greater exclusion (3.0-3.5 ml) from the gel ($K_{av} = 0.297 \pm 0.004$ S.D.; $p < 0.001$). The elution chromatogram for serum TSH was essentially the same before and after TRF. A clear difference between pituitary and circulating TSH has been demonstrated by gel chromatography. Further investigation will be required to determine whether this difference is related to the biosynthesis, secretion, or peripheral metabolism of pituitary TSH or to its alteration during extraction and purification.

Hormonal regulation of lipolysis in animal tissue is due to the activation of triglyceride lipase. Lipolytic hormones such as epinephrine and ACTH, activate adenyl cyclase and increase intracellular cAMP levels. Cyclic AMP activation of hormone-sensitive lipase requires ATP and is due to cAMP stimulation of protein kinase. Hormone-stimulated lipolysis in human adipose tissue is felt to have a similar mechanism. In human adipose cells adenyl cyclase is activated with an increase in intracellular cAMP during hormone-stimulated lipolysis. It has been shown

that a partially purified lipase from human adipose tissue is stimulated by cAMP in the presence of ATP and $MgCl_2$. This stimulation is blocked by a heat stable protein inhibitor which is specific for cAMP stimulated protein kinase. Partial purification and characterization of cAMP-stimulated protein kinase from human subcutaneous breast and pannicular adipose tissue were accomplished in this laboratory. Tolbutamide, an antilipolytic drug, has been previously shown to inhibit protein kinase from rat and bovine adipose tissue and our laboratory has confirmed these studies for human adipose tissue. The isolation and characterization of protein kinase from human adipose tissue provides additional evidence for the possibility that the mechanism of action of hormone-stimulated lipolysis in humans is mediated by protein kinase.

Plasma prolactin levels were measured in a group of fourteen young men undergoing their first parachute experience at the Infantry School at Fort Benning, Georgia. Basal prolactin levels were determined on three occasions prior to the first parachute jump between 0600 and 0700 hours and 20 to 30 minutes before jumping at approximately 1300 hours; mean basal prolactin levels were similar on all occasions and did not increase significantly as the time of the first jump approached. However, mean plasma prolactin levels had increased significantly over all basal samples ($p < 0.005$) within several minutes of landing in the drop zone. Thus, the apprehension of an initial parachute jump stimulates a significant plasma prolactin release in a situation controlled to eliminate physical stress and plasma prolactin levels may be useful as a marker for the presence of apprehension.

	Day -13 0600	Day -3 0600	Day 0 0600	Day 0* 1300	Day 0** 1400
Mean HPr	8.69	8.73	10.25	10.37	19.30
SEM	0.73	0.85	1.35	1.16	3.10
n	14	14	14	14	14
P value of time 1400	p < 0.005		< 0.005	< 0.005	
Day 0					
Compared to:					

*Approximately 30 minutes before jumping from aircraft.

**Approximately 10 minutes after jumping from aircraft.

To determine if the secretory mechanism in parathyroid adenomas is independent of blood calcium and autonomous, EDTA and calcium infusions have been carried out in 10 patients with primary hyperparathyroidism who had an adenoma, three patients with hyperplasia, and one patient with tertiary hyperparathyroidism. In 8 of the 10 patients who had an adenoma, immunoreactive PTH and cyclic AMP were not autonomous and in two patients secretion appeared to be autonomous. The three patients with hyperplasia, all showed non-autonomy. The one patient with tertiary hyperparathyroidism received only an EDTA infusion and showed marked increase in iPTH and urinary cyclic AMP. It can be concluded that PTH secretion from an adenoma is usually not autonomous as was previously believed. The cyclic 3' 5' AMP findings also support this conclusion. In six patients with idiopathic hypercalciuria, the urinary cyclic 3' 5' AMP varied inversely with plasma ionized and total calcium. The rapid responses of cyclic 3' 5' AMP allow the conclusion that the biological half life of plasma PTH is very short. Urinary cyclic 3' 5' AMP is rapidly suppressed by calcium infusion in both idiopathic hypercalciuria and adenomatous hyperparathyroidism.

Observation of different values of parathyroid hormone in the same patient's serum or plasma suggested a study to determine the effect of heparin on the radioimmunoassay of parathyroid hormone (PTH). The assay was performed using ^{125}I -labelled bovine PTH (^{125}I -bPTH) and a guinea pig antiovine antibody. Partially purified human PTH was used as standard. Phase separation was accomplished by dextran-coated charcoal. In 19 normal volunteers serum and plasma samples obtained simultaneously were analyzed in the same assay. Only 2 of the 19 plasma samples had detectable PTH while 8 of the 19 serum samples were detectable. The serum PTH values were significantly higher than the plasma values ($p < 0.01$). In other assays using three different ^{125}I -bPTH tracers as many as 15 of 18 sera of normal volunteers had detectable PTH, while only 5 of the simultaneously obtained plasma were detectable. The addition of heparin to serum samples (1.25 U/100 μl) similarly depressed the immunoassayable PTH (iPTH) levels ($p < 0.05$). As little as 1.4 U heparin added to the partially purified human PTH standards also depressed the iPTH level compared to the same amount of standard not subjected to heparin. Gel filtration studies using a P-60 Bio-gel column revealed that the addition of heparin to ^{125}I -bPTH resulted in a persistent shift of the elution point toward the void volume. This suggests binding of the heparin to the PTH resulting in lower values of iPTH in plasma compared to serum samples.

3. Steroid Metabolism.

A Porter-Silber procedure, a fluorometric procedure, a double-isotope derivative procedure and a competitive protein-binding assay procedure were employed for the determination of plasma 17-OH corticosteroids.

Project 3A062110A822 MILITARY INTERNAL MEDICINE

Task 00, Military Internal Medicine

Work Unit 120, Metabolic response to disease and injury

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION ^a	2. DATE OF SUMMARY ^a	REPORT CONTROL SYMBOL	
				DA OA 6445	73 07 01	DD-DR&E(AR)636	
3. DATE PREV SUMMARY ^a	4. KIND OF SUMMARY	5. SUMMARY SCTY ^a	6. WORK SECURITY ^a	7. REGRADING ^a	8A. DISSEM INSTRN ^a	8B. SPECIFIC DATA- CONTRACTOR ACCESS ^a	9. LEVEL OF SUM A. WORK UNIT
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10. NO./CODES ^a	PROGRAM ELEMENT	PROJECT NUMBER		TASK AREA NUMBER		WORK UNIT NUMBER	
A. PRIMARY	62110A	3A062110A822		00		121	
B. CONTRIBUTING							
C. DISSEM INSTRN ^a	CDOG 114 (f)						
11. TITLE (Precede with Security Classification Code) ^a							
(U) Pathogenesis of Enteric Disease							
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010100 Microbiology							
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20. RESPONSIBLE DOD ORGANIZATION				20. PERFORMING ORGANIZATION			
NAME ^a : Walter Reed Army Institute of Research				NAME ^a : Walter Reed Army Institute of Research			
ADDRESS ^a : Washington, D. C. 20012				ADDRESS ^a : Div of CD&I Washington, D. C. 20012			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)			
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21. GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER: [REDACTED]			
Foreign Intelligence Not Considered				ASSOCIATE INVESTIGATORS			
				NAME: Gemski, P.			
				NAME: DA			
22. KEYWORDS (Precede EACH with Security Classification Code)							
(U) Diarrhea; (U) Dysentery; (U) Bacillary; (U) Salmonellosis; (U) Immunity; (U) Immunization							
23. TECHNICAL OBJECTIVE ^a , 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
<p>23. (U) To find improved procedures to control diarrheal disease. Present work involves the preparation and testing of oral vaccines against bacillary dysentery, the identification and characterization of agents involved in travelers diarrhea, and the virulence of Salmonella.</p> <p>24. (U) Attenuated dysentery strains are being developed. They are being evaluated for safety in several systems and are being tested for potency in monkeys and in man.</p> <p>25. (U) 72 07-73 06 A new E. coli hybrid expressing the somatic determinants of S. flexneri 2a has been employed as a vaccine in volunteers. Dosages of ten to the tenth cells in volunteers have produced no illness and in preliminary challenge experiments two doses provided some degree of protection against shigellosis. Additional hybrids with other shigella serotypes are now being prepared. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 72 - 30 Jun 73.</p>							

^a Available to contractors upon originator's approval.

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AND 1 28-1 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE

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Project 3A062110A822 MILITARY INTERNAL MEDICINE

Task 00, Military Internal Medicine

Work Unit 121, Pathogenesis of enteric diseases

Investigators.

Principal: Samuel B. Formal, Ph.D.

Associate: Peter Gemski, Jr., Ph.D.

Description.

The pathogenesis of enteric infections, particularly those caused by Shigella and Salmonella, is being studied to establish factors and mechanisms by which disease is provoked. Through an elucidation of such elements, procedures for prevention and control of diarrheal diseases can be devised.

Progress.

1. The virulence of Salmonella typhimurium with reference to its lipopolysaccharide (LPS) structure

a. In the pathogenesis of salmonellosis, the capacity of Salmonella strains to provoke disease is related to three broad bacterial attributes, (a) the capacity to invade the gastrointestinal mucosal layer, (b) an ability to cause fluid secretion by the bowel, which results in diarrhea and (c) the capacity to disseminate from the intestinal site of infection to various vital organs, this being a consequence of the organisms ability to survive and multiply within the reticuloendothelial system (RES) of the infected host. Much of the previous work on virulence of Salmonella strains is based on the mouse laboratory model, in which LD₅₀ determinations after intraperitoneal challenge served to estimate the degree of virulence of an organism. Although such a laboratory model appears adequate for distinguishing between strains of different virulence, it is apparent that this model may not reflect the capacity of an organism to invade the intestinal mucosa and to produce diarrhea. Indeed, it is likely that the intraperitoneal challenge of mice represents a model for assessing the ability of an organism to survive and multiply in the RES of this species. Thus, much of the previous work on Salmonella virulence has not attempted to investigate bacterial factors specifically concerned with the initial steps of pathogenesis, i.e. mucosal invasion and subsequent fluid accumulation. As a consequence, we have initiated studies during the past year on the relationship of LPS structure to the capacity of Salmonella typhimurium to invade mucosa and evoke fluid accumulation. We employed the ligated rabbit ileal loop as an animal model in these experiments.

Recent studies performed in conjunction with the Gastroenterology Department, Division of Medicine, WRAIR (see Annual Report, 1973) have established that the ligated rabbit ileal loop can serve as a sensitive model for studying the capacity of S. typhimurium to invade mucosa, evoke fluid accumulation, and translocate across the intestinal wall.

b. S. typhimurium strain TML, isolated from a patient with severe salmonellosis, served as the parental strain in this study. This strain behaves as a typical S. typhimurium in biochemical tests. In addition, on the basis of serology and phage sensitivity, it is fully smooth and thus is capable of synthesizing a complete core and O-repeat unit LPS complex. In the mouse laboratory model, strain TML possessed an LD₅₀ of about 10-100 cells. The behavior of TML in the rabbit ileal loop model further confirmed its highly virulent characteristics. Ligated ileal loops (one test loop and one broth loop with an intervening blank loop per animal) were constructed in starved rabbits. One ml of an overnight broth culture of TML was inoculated into test loops and after 18 hrs., the animals were sacrificed, examined for fluid accumulation in infected loops and then subjected to routine autopsy procedures. Strain TML consistently evoked fluid secretion in the infected ligated loop. Histological examination of such infected ileum revealed TML to be highly invasive, with bacterial cells present in the epithelium and lamina propria. Additional evidence for the high invasiveness of strain TML was provided by the recovery to TML from the heart, liver and spleen of infected rabbits, thus indicating that mucosal penetration subsequently led to dissemination of the pathogen to various vital organs.

c. Because this animal model of salmonellosis enabled us to distinguish some of the steps in pathogenesis, we next decided to alter the LPS properties of the pathogen and to assess the effects of such a change on pathogenesis. S. typhimurium TML, grown overnight in broth, was seeded on nutrient agar plates and then spotted with a mixture of phages capable of infecting only smooth S. typhimurium strains. After overnight incubation at 37°C, clones of strain TML resistant to these phages were isolated. Two derivatives, termed 47B2 and 47A5, were chosen for further study and found to be spontaneous rough mutants of S. typhimurium TML. On the basis of their sensitivity to a battery of phages specific for rough strains, it became apparent that strains 47B2 and 47A5 were rough B mutants (rfb). Extensive studies on the genetic control of Salmonella LPS have indicated at least two broad classes of mutants, termed rough A (rfa) and rough B (rfb). The genes of rfa class, located near the xylose fermentation gene(s) of S. typhimurium, have been shown to control the biosynthesis of the core of the LPS layer. Although rfa mutants are capable of synthesizing the O-repeat unit of the LPS layer, they are unable to attach these serologically specific sugar determinants to a defective core and hence are rough.

In contrast, the genes of the rfb cluster, shown to map near the histidine biosynthesis locus of S. typhimurium, control the biosynthesis of the O-repeat unit structure of the LPS layer. Thus mutants of the rfb class, although able to synthesize a fully functional LPS core, are rough as a consequence of some defect in the steps of the O-repeat unit synthesis. Since mutant strains 47B2 and 47A5 yielded a phage sensitivity pattern typical for the rfb LPS mutant type, we infer that these two rough derivatives are capable of producing a complete LPS core, but are unable to synthesize a functional O-repeat component. In the case of mutant 47B2, genetic crosses were employed to confirm that the mutation was of the rfb type. By means of mutagenesis with N-methyl-N-nitro-nitrosoguanidine, a xylose⁻, histidine⁻ derivative of 47B2 was constructed and employed as a recipient in matings with a smooth S. typhimurium Hfr donor. 64 xyl⁺ 47B2 recombinants (selected for the inheritance of xylose⁺ utilization genes from the donor) and 100 his⁺ 47B2 hybrids (selected for inheritance of donor histidine⁺ genes) were then analyzed by phage sensitivity testing to determine whether they were of a rfb or smooth phenotype. A high incidence of smooth recombinants among the his⁺ hybrids would be expected if derivative 47B2 is a rfb mutant, since this locus has been positioned near the histidine operon. The results of these hybridization experiments fully support our conclusion that 47B2 is altered in the rfb locus. Eighty-five of 100 his⁺ recombinants were found to be smooth, while none (0/64) of the xyl⁺ (rfa region) hybrids were smooth, all retaining their rough phenotype.

c. The S. typhimurium parent strain, TML, and the two rough derivatives 47B2 and 47A5 were next compared for their virulence in the mouse model (to determine LD₅₀) and the rabbit ileal loop model. As summarized in Table 1, both of the rough derivatives have been markedly altered in their LD₅₀ value for mice ($> 10^8$ cells) as compared to the parent strain, TML (102 cells). It is also evident that both 47B2 and 47A5 were unable to provoke fluid accumulation in the ligated loop. Cytological examination, however, revealed significant invasion of the mucosa by both 47B2 and 47A5 a necessary prerequisite for evoking fluid accumulation. In addition, the capacity for dissemination from the ileum appears reduced in the rough derivatives. Unlike the smooth parent, TML, which consistently disseminated to other vital organs in this animal model, both rough derivatives (47B23 and 47A5) appeared variable in this regard. In some experiments, no pathogens were recovered from either the heart, liver or spleen; in many experiments, only the spleen proved positive for pathogens.

d. These observations suggest that a single step mutation altering the synthesis of LPS in S. typhimurium results in a loss in the capacity of the organism to induce fluid accumulation. Although invasiveness of such rough derivatives is not altered significantly as evidenced by

our histological findings, 47B2 and 17A5 appear to be altered in their ability to survive and disseminate. This finding is not unexpected, since rough derivatives are more sensitive to host defense mechanisms (RES, etc.)

e. The possible involvement of the LPS layer in the capacity of S. typhimurium to provoke a positive ileal loop reaction is further supported by genetic hybridization experiments. Strain 47B2, one of the rough mutants unable to evoke fluid accumulation, was mated with a smooth S. typhimurium Hfr donor and his⁺ recombinants were recovered. A high proportion of these his hybrids were smooth (based on phage sensitivity), a result of inheriting rfb⁺ genes from the donor. When such smooth his⁺ hybrids were tested in the rabbit ileal loop model, over 75% regained the capacity to evoke fluid accumulation. All of the rough his⁺ hybrids, similarly tested, were unable to cause a positive loop reaction. These findings thus indicate that a genetic repair of the rfb lesion in mutant 47B2 can lead to a return of the fluid provoking property.

f. In an effort to define the precise role of LPS in this step of pathogenesis, studies are being directed to other classes of rough mutants of strain TML.

2. Oral living shigella vaccines

a. One aspect of our research program on diarrheal diseases involves the development of oral, attenuated vaccines against bacillary dysentery. Previous studies in this department have been based on the concept of preparing avirulent hybrids and mutants of Shigella flexneri for possible vaccine strains (see previous annual reports). Although some of these strains were shown to be safe and significantly effective in limited human volunteer studies performed by the University of Maryland Vaccine Development Group, it is impossible to obviate the theoretical possibility that these strains could revert to a virulent state.

b. In an effort to eliminate such complications with the safety of living oral vaccines, we have taken an alternative approach for preparing possible vaccine candidate strains. In principle, our approach has been to alter, by genetic manipulations, avirulent E. coli strains so that they now express the group and type-specific antigens of Shigella rather than their native E. coli antigenic complex. Since the E. coli parent strains are natively avirulent, the problems of reversion to virulence by hybrid vaccine strains appear non-existent. The feasibility of preparing such intergeneric E. coli hybrids is a direct consequence of our investigations (see previous annual report) on the genetic control of Shigella antigens which identified the chromosomal

location and functions of genes controlling both type and group antigens of Shigella flexneri.

3. Construction of E. coli hybrids which express Shigella flexneri 2a group and type antigens

As described in previous annual reports, the 3,4 group antigen gene(s) of S. flexneri 2a are close to the histidine locus whereas the type 2 gene(s) are distal to this group locus, being positioned between the proline and lactose genetic loci. As a consequence, E. coli hybrids expressing these antigens had to be constructed by "back-crossing" experiments. The donor strain was S. flexneri 2a Hfr 256 and behaves serologically as a typical S. flexneri 2a. As recipients we employed two E. coli strains, E. coli K-12 strain 1133 and E. coli O3 strain RJ91, which did not express any virulent properties in a number of animal models. In order to introduce the genes for Shigella 3,4 group antigen, the Hfr 256 donor was mated with both recipient lines and selection was made for hybrids which inherited the donor his⁺ genes. A high proportion of such E. coli hybrids were found to inherit and express the S. flexneri 2a group 3,4 antigen.

b. Two of these E. coli hybrids were selected for additional hybridization experiments. The E. coli K-12 1133 his⁺ 3,4, group factor⁺ hybrid was remated with the S. flexneri 2a Hfr 256 donor, selecting this time for the inheritance of the proline, type-antigen chromosomal segment. On the basis of genetic and serological analyses, one such hybrid was chosen as a possible vaccine strain. E. coli K-12 hybrid 1133 his⁺ pro⁺ was found to be stable and behaved serologically as a typical S. flexneri 2a, expressing both the group 3,4 and type-specific 2 antigen antigen. By similar procedures, an E. coli RJ91 hybrid expressing S. flexneri 2a antigens was also prepared. Since both E. coli recipient lines are natively avirulent, no problem with reversion to virulence by these hybrids was anticipated. Preliminary tests on human volunteers in conjunction with Dr. H. L. Dupont of the University of Maryland have shown these hybrid strains to be safe at dose levels of 1×10^{11} cells and that protection to challenge by virulent S. flexneri 2a can be achieved with two immunizing doses.

c. Construction of E. coli hybrids expressing other S. flexneri group and type-specific antigens

a. The promising results on the use of E. coli hybrids with S. flexneri 2a antigens as a live vaccine led us to expand our investigations to other S. flexneri serotypes. To achieve this, we have in recent months given top priority to the construction of Hfr donor strains in the other serotypes of S. flexneri, such strains being essential for the construction of E. coli hybrids of appropriate serotypes. By employing procedures similar to those described in previous annual reports for preparing a S. flexneri 2a donor, we now have available Hfr donor strains of S. flexneri serotypes 1a, 1b, 3a, and 4b in

addition to the original 2a donor. Our efforts to prepare Hfr derivatives of S. flexneri serotypes 5 and 6 have not been successful as yet. Preliminary mating experiments with these new S. flexneri Hfr strains indicate that they are capable of transferring chromosomal genes to E. coli K-12, thus making it feasible to construct E. coli hybrids with different S. flexneri type antigens.

b. In addition, we are continuing studies on the genetic control of S. flexneri group antigens. Besides the 3,4 group antigen (already discussed) some S. flexneri serotypes express group antigen 6 and group antigens 7,8,9. We have recently isolated a temperate bacteriophage from S. flexneri 3a which is capable of achieving group antigen 6 conversion. Lysogenization of S. flexneri strains with this phage results in a conversion of the group 3,4 determinants to a group 6 serological determinant. Of particular relevance to our vaccine development program is the finding that E. coli hybrids with the S. flexneri group 3,4 antigen can also be converted to group factor 6, to yield derivatives expressing both the 3,4 and 6 group antigens. This finding suggests the feasibility of constructing a vaccine strain which may stimulate immune response to the two major group antigens typical of S. flexneri.

4. Studies on Invasive E. coli

a. On November 1971 a social affair took place at WRAMC. Twenty-eight of thirty-seven persons attending this party developed acute dysentery 24-48 hours after eating contaminated imported French Camembert cheese. MAJ E. Frank Tulloch studied this outbreak. An O:124 Escherichia coli was isolated from the cheese and from the stools of nine ill individuals. Biological testing revealed this organism to be invasive in character. Predominant symptoms included fever, malaise, tenesmus, abdominal cramping and diarrhea. None of the patients required hospitalization or antibiotic therapy and most were asymptomatic within one week. This episode was part of the first known outbreak of invasive E. coli dysentery in adults occurring in the United States.

Summary and Conclusions

1. The pathogenicity of Salmonella typhimurium with particular reference to invasiveness and fluid accumulation as factors in virulence has been studied in the ligated ileal loop model (Rabbit). An S. typhimurium strain (mouse LD₅₀ 10¹) was found to induce positive fluid accumulation in the ligated loop and to invade subsequently into the heart, liver and spleen.

A single step mutation, altering the synthesis of lipopolysaccharide (LPS) results in a loss of the capacity of the organism to induce a positive loop reaction, but does not significantly alter the invasiveness of the organism. The findings indicate that the invasive property of S. typhimurium may not be dependent upon the presence of a complete LPS layer.

2. An aspect of our program on diarrheal diseases involves the development of oral, attenuated vaccines against bacillary dysentery. Previous studies have been based on the concept of preparing avirulent hybrids and mutants of Shigella flexneri for possible vaccine strains.

As an alternative approach, we have recently constructed by intergeneric hybridization techniques E. coli hybrids which now express the group and type-specific antigens of S. flexneri 2a rather than their native antigens. Since the original E. coli parents are natively avirulent, the problem of reversion to virulence by hybrid vaccines is obviated. Preliminary tests on human volunteers in conjunction with the University of Maryland have shown that the vaccine is safe at dose levels of 10^{11} cells/ml and that protection to challenge by virulent S. flexneri 2a can be achieved with two immunizing doses.

Hfr derivatives of different S. flexneri serotypes have been constructed for use in the preparation of E. coli hybrids of various S. flexneri serotypes. In addition, continuing studies on the genetic control of lipopolysaccharide antigenic determinants of Shigella flexneri have resulted in the isolation of a temperate bacteriophage which has the property of antigenic conversion. Lysogenization of host strains with the S. flexneri group antigens (3,4) results in antigenic conversion to group factor 6. Preliminary studies with this phage indicate that it will be useful in constructing additional E. coli hybrids with S. flexneri antigen determinants for possible use as oral vaccines.

3. An outbreak of diarrhea occurred in WRAMC personnel which was caused by cheese infected with invasive E. coli O-124.

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Table 1. Virulence of *S. typhimurium* TML and its Derivatives

Strain	LD ₅₀ (mice) # cells	Rabbit ileal loop		
		fluid accumulation	mucosal invasion	dissemination across intestinal wall
TML	10 ²	+	+	+
47B2 <u>rfb</u>	> 10 ⁸	-	+	±
47A5 <u>rfb</u>	> 10 ⁸	-	+	±

Project 3A062110A822 MILITARY INTERNAL MEDICINE

Task 00, Military Internal Medicine

Work Unit 121, Pathogenesis of enteric diseases

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION ^a	2. DATE OF SUMMARY ^a	REPORT CONTROL SYMBOL	
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11. TITLE (Precede with Security Classification Code) ^a							
(U) Microbial Genetics and Taxonomy							
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010100 Microbiology							
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17. CONTRACT GRANT				18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	
a. DATES/EFFECTIVE: NA				PREVIOUS		b. FUNDS (in thousands)	
b. NUMBER ^a				FISCAL		73	
c. TYPE:				YEAR		3	
d. KIND OF AWARD:				CURRENT		120	
				74		3	
						120	
19. RESPONSIBLE DOD ORGANIZATION				20. PERFORMING ORGANIZATION			
NAME: Walter Reed Army Institute of Research				NAME: Walter Reed Army Institute of Research			
ADDRESS: Washington, D. C. 20012				ADDRESS: Division of Communicable Disease and Immunology			
				Washington, D. C. 20012			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)			
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				SOCIAL SECURITY ACCOUNT NUMBER			
21. GENERAL USE				ASSOCIATE INVESTIGATORS			
Foreign Intelligence Not Considered				NAME: Wohlhieter, Dr. J.A.			
				NAME: Johnson, Dr. E.M.			
22. KEYWORDS (Precede EACH with Security Classification Code)							
(U) Microbial Genetics; (U) Vaccine; (U) Enteric Bacteria; (U) Antigens (U) Virulence;							
(U) Salmonella; (U) Drug Resistance							
23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
<p>23. (U) Definition in genetic and molecular terms of the properties of gene transfer antigenicity, and virulence of pathogenic enteric bacteria which because of their disease producing capabilities, are of importance to military medicine concerned with the prevention and treatment of such infections in Army personnel. We anticipate that it will be possible to genetically modify enteric bacteria to any desired antigenic structure and pathogenicity to serve as vaccine strains or as tools to study the infectious process.</p> <p>24. (U) Use of genetic recombination between strains of enteric bacteria. Where possible, the genetic results are extended to include study of the informational macromolecules involved.</p> <p>25. (U) 72 07-73 06 We have successfully employed mouse virulent Salmonella typhimurium hybrids expressing the S. typhosa antigen 9, d, and Vi as challenge organisms in Swiss white mice to determine the degrees of protection conferred by immunization with heat killed, alcohol treated and acetone treated typhoid vaccines. In our continuing investigation of the behavior of foreign DNA in bacterial hosts, we have determined that extrachromosomal conservation of S. typhimurium DNA as supercoiled circular molecules in partially diploid Escherichia coli hybrids requires physical association of the DNA fragment with some part of the E. coli sex factor, F. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 72 - 30 Jun 73.</p>							

^a Available to contractors upon originator's approval

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Project 3A062110A822 MILITARY INTERNAL MEDICINE

Task 00, Military Internal Medicine

Work Unit: 122, Microbial Genetics and taxonomy

Investigators.

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Description.

1. Mouse virulent Salmonella typhimurium hybrids expressing the S. typhosa antigens 9, d, and Vi were employed as challenge organisms in Swiss white mice to determine the degrees of protection conferred by immunization with heat-killed, alcohol-treated, and acetone-treated typhoid vaccines.

2. The extrachromosomal conservation of Salmonella typhimurium deoxyribonucleic acid in a supercoiled, circular configuration in partially diploid hybrids of Escherichia coli was found to require physical association of the DNA fragment with at least some part of the E. coli sex factor F.

3. Hybrids have been constructed between coliphage lambda and Salmonella phage P22 through the use of a Salmonella typhimurium hybrid sensitive to these phages.

Progress.

1. Assay of typhoid vaccines with Salmonella typhosa-Salmonella typhimurium hybrids

a. A number of investigators have shown that the active mouse protection test is unable to differentiate between the effectiveness of various typhoid vaccines for man. This lack of a meaningful, laboratory biological assay has handicapped both the selection and improvement of typhoid vaccines. Salmonella typhosa, highly pathogenic for man, exhibits only minimal virulence for experimental animals. It takes approximately 5×10^7 organisms to kill a mouse in 24 to 48 hr. and the death is due to toxemia rather than to infection. This may be the main reason why the results of animal potency tests do not reflect the value of typhoid vaccines for preventing typhoid in man.

b. A number of salmonellae, such as S. typhimurium, S. enteritidis, and S. moscow, are natural pathogens for the mouse, producing a systemic disease leading to septicemia which resembled typhoid infection in humans. We therefore considered the possibility of overcoming the inadequacy of S. typhosa as a challenge strain by producing, by conjugation between S. typhosa and S. typhimurium, a hybrid strain with the antigenic structure of S. typhosa and the pathogenicity (for the mouse) of S. typhimurium.

c. S. typhimurium WR5004 was mated with the S. typhosa Hfr donor WR4000, and WR5004 hybrids were selected for receipt of his⁺ marker of the donor. As we have shown previously, the majority of his⁺ selected S. typhimurium hybrids obtained from mating with S. typhosa WR4000 also inherit the closely linked genetic determinant of somatic antigen 9. Because the genetic determinant of this antigen is the allele of the somatic antigen 4 determinant of S. typhimurium, recombination of this locus results in loss of expression of antigen 4 by those S. typhimurium recombinants which express antigen 9. In addition, such recombinants also lose antigen 5 because this antigen cannot be expressed in strains lacking the determinant of antigen 4.

d. The genetic locus of the phase 1 flagellar antigen determinant of S. typhosa is sufficiently close to the his⁺ locus that some of the his⁺-selected hybrids of S. typhimurium undergo recombination at that locus as well. Inasmuch as the phase 1 flagellar antigens of all Salmonella species comprise an allelic series at this locus, we were able to obtain his⁺ hybrids of S. typhimurium WR5004 which, in addition to antigen 9, expressed also the phase 1 flagellar antigen d of S. typhosa. As in the case of the somatic antigen, such hybrids lose their native phase 1 flagellar antigen, i.

e. The genetic determinant of the Vi antigen, viaB, was introduced into two of the S. typhimurium WR5004 his⁺ hybrids (one expressing S. typhosa antigen 9 and the other expressing S. typhosa antigens 9 and d) by mating with the Vi antigen-expressing S. typhimurium Hfr WR4010. Because of the considerable genetic map distance between the viaB locus and the nearest available selective marker in the S. typhimurium WR5004 hybrids (ara⁻), it was necessary to examine about 200 ara⁺ selected recombinants in each case to obtain a hybrid expressing the Vi antigen. As described below each of the two Vi-expressing hybrids, as well as the hybrid expressing only the 9 antigen of S. typhosa, retained the virulence of the S. typhimurium WR5004 recipient.

f. 16- to 18-g C57 mice bred at the Laboratory Center for Disease Control, Ottawa, Ontario, Canada, were used. Groups of 20 mice were infected intraperitoneally with graded doses of bacteria in a 0.5 ml volume. The mouse lethal dose (MLD) was less than 50 bacteria for each hybrid. Anatomopathological and bacteriological

findings revealed that the hybrids caused both systemic infection and septicemia, with lesions appearing in several organs, especially the liver. The hybrids had therefore retained the pathogenicity of the recipient parent strain S. typhimurium WR5004 and caused a true infection with a small challenge dose, thereby fulfilling a requirement for the establishment of a model typhoid infection. Similar results were obtained using the LCDC strain of Swiss white mice (P.E.T.). All of the hybrids remained stable with regard to their inherited biochemical and antigenic characters; no loss or reversal of any of these characters was observed during infection in the mouse.

g. The ability of S. typhosa vaccines to protect against challenge was determined by immunizing intraperitoneally C57 mice with a dose of 0.5 ml of one of the three types of S. typhosa vaccine and by challenging 14 days later with 10 MLD of one of the hybrids. It was found, however, that this immunization, although prolonging the life of the mice, did not confer complete protection, as all of the mice died within the 21-day period of the experiment. The procedure was then repeated with the LCDC-bred Swiss white mice (P.E.T.), and again challenging with 10 MLD. With this breed of mice a protective effect was observed over the full 21 days against challenge by each of the hybrid strains. The results of this experiment are shown in Table 1. The least degree of protection was afforded by the heat-killed, phenol-preserved vaccine, which proved inferior in this regard to both acetone- and alcohol-treated products. None of the vaccines protected the mice against challenge by the parent S. typhimurium.

h. The consistently higher protection which we observed with the acetone-treated vaccine is in keeping with clinical trials with humans. The lower level of protection afforded by the heat-killed vaccine, as compared to the alcohol vaccine, is not in accordance with the results of reported field trials. This factor has to be studied since it is possible that our alcohol vaccines could have been substantially different from those used in the field trials.

i. The observation that the typhoid vaccines employed here were protective against the hybrid S. typhimurium strains which expressed S. typhosa antigens, but not against the S. typhimurium parent strain, is in agreement with the predicted results if S. typhosa antigens are indeed involved in the immune mechanism. No significant differences were observed in our assay results among the challenge strains of S. typhimurium hybrids whether they contained only factor 9; 9, Vi; or the 9, Vi, d antigens. However, it would be premature at this stage to draw any conclusions with regard to the roles of the

TABLE 1. Representative data from mouse protection tests employing *Salmonella typhosa* vaccines

Challenge strains	Survivors/challenged mice after 21 days			
	Acetone treated ^a	Alcohol treated	Heat-killed	No vaccine (control)
<u>Salmonella typhimurium</u> WR5004 parent strain	5/50	6/50	4/48	5/50
<u>S. typhimurium</u> hybrid WR5004 His+ 9,12:i-1,2	32/49 ^b	24/50	12/50	4/48
<u>S. typhimurium</u> hybrid WR5004 His+ Ara+ -1 9, 12, Vi:1-1,2	33/50 ^b	32/50	14/50	4/48
<u>S. typhimurium</u> hybrid WR5004 His+ Ara+ - 2 9,12, Vi:d-1,2	33/50 ^b	27/50	18/50	4/50

^a

Salmonella typhosa vaccines. Dose: 0.5 ml of undiluted vaccine given intraperitoneally.

^b

Acetone-treated vaccine was significantly more protective than the heat-killed product ($P < 0.05$).

individual S. typhosa antigens, and in fact, these experiments were not designed with that aim in mind.

j. Finally, it should be noted that the selection of the mouse strain for this assay was of critical importance. The virulence properties of the hybrids appeared to be identical in both the C57 mice and the Swiss white mice, but there was a marked difference in the ability of the vaccines to protect the two mouse strains against challenge with these same hybrids. Protection was effective for the Swiss white mice over the 21-day observation period, but not for the C57 mice. All of the latter died, and the immunization served only to bring about a delay in death. This would suggest that the C57 mouse lacks the ability to develop a full immunological response against the challenge organisms.

2. Conservation of Salmonella typhimurium deoxyribonucleic acid in partially diploid hybrids of Escherichia coli

a. In a recent attempt (see Annual Report, WRAIR, 1971) to gain some knowledge of the manner in which supernumerary genes are conserved in partially diploid bacterial cells, we examined unstable Salmonella typhosa hybrids derived from matings with Escherichia coli K-12 Hfr strains. Some of these S. typhosa hybrids were found to contain supercoiled circular deoxyribonucleic acid (DNA) molecules, whose presence was correlated with the presence of the added E. coli genetic material. That is, segregant cells from which E. coli genes had been lost were found also to have lost the circular DNA. The possibility that the conservation of these E. coli genes in a circular configuration might have been the consequence of their association with the sex factor, F, was not indicated by tests of hybrid donor ability or adsorption of male-specific bacteriophage.

b. With regard to its susceptibility to those presently known phages which might be employed to detect the presence of F, S. typhosa is severely limited. Male-specific phages do not form visible plaques on male derivatives of S. typhosa, as they do on E. coli K-12 males, and the sensitivity of F-containing S. typhosa strains to these phages must be demonstrated by experiments which involve an increase in titer. As for female-specific phages, we do not know of any to which S. typhosa is sensitive, whereas a number of such phages are available which lyse E. coli K-12. We therefore examined heterozygous, partially diploid E. coli K-12 hybrids derived from mating with an S. typhimurium Hfr strain. The hybrids were tested with respect to their patterns of susceptibility to the male-specific phage R-17, and the female-specific ØII, and were examined for the presence of supercoiled circular DNA by the dye-buoyant density method. Our findings

in this mating system indicated that when the added DNA in a diploid hybrid is maintained in a circular configuration, at least some part of the sex factor, F is involved in that maintenance.

c. The transfer origin of S. typhimurium Hfr WR4016 is situated between the xyl and malA chromosomal loci of this organism, xyl⁺ being the marker which is transferred early, and mal⁺ the terminal marker linked to F. In a mating of S. typhimurium WR4016 with the E. coli K-12 recipient strain WR3026, E. coli hybrids selected for receipt of the Salmonella xyl⁺ character were recovered at a frequency of 5×10^7 per donor cell. Of 100 xyl⁺ hybrids examined, 40 expressed also the tna⁻ character of the donor. This expression of a recessive allele indicates, of course, that recombination of S. typhimurium DNA did occur in these hybrids. On the other hand, among those xyl⁺ hybrids in which recombination of the tna⁻ marker was not observed, 37 were seen to segregate daughter cells from which the inherited Salmonella xyl⁺ marker had been lost.

d. Of the 37 partially diploid hybrids which were detected, 28 were found to have acquired sensitivity to the male-specific phage, R-17. In fact, 84 hybrids, overall, acquired R-17 sensitivity in this mating, an indication of instability (reversion to F⁺) of the Hfr donor. It was anticipated that the mal⁺ character of the 5 R-17 sensitive, xyl⁺ mal⁺ hybrids (which like the xyl⁺ character, was unstable) would prove to be associated with F. Upon examination of these hybrids, it appeared, in fact, as if both the xyl⁺ and mal⁺ markers were part of the same F-merogenote. Conjugal transfer of either marker from each of these hybrids to the E. coli recipient strain WR3030 occurred at a frequency of $1-2 \times 10^{-2}$ per donor cell. All recipients of the xyl⁺ marker were found also to have received the mal⁺ character, and all which received mal⁺ as the selected marker inherited the xyl⁺ character as well. Segregant cells (from each of the five xyl⁺ mal⁺ hybrids) from which the xyl⁺ marker was lost also become mal⁻, and mal⁻ segregants invariably were found to have lost the xyl⁺ marker.

e. We examined also, with respect to their ability to transfer the xyl⁺ marker to E. coli WR3030, three diploid, R-17 sensitive xyl⁺ ilv⁺ hybrids and 10 diploid R-17 sensitive, xyl⁺ hybrids. In sharp contrast to the high frequency transfer observed with the xyl⁺ mal⁺ hybrids, xyl⁺ transfer by these 13 hybrids occurred at frequencies of 10^{-5} to 5×10^{-6} per donor cell. In this instance, therefore, the hybrids behaved in the manner of F⁺ donors, rather than as F-prime strains.

f. Nine partially diploid hybrids were obtained which did not exhibit sensitivity to R-17. Neither as expected, did they show any capability of transferring the xyl⁺ marker in mating attempts with E. coli WR3030. When examined for their pattern of susceptibility to the female specific phage ØII, however, 6 of these hybrids inhibited the development of phage plaques in the manner characteristic of F-containing derivatives of E. coli WR3026. Several xyl⁻ segregants obtained from each of the 6 R-17 non-sensitive xyl⁺ diploid hybrids which had exhibited the ØII male response were tested with ØII and all of these were observed to respond as females. Thus, loss of the xyl⁺ marker was accompanied, in these hybrids, by loss of their male response to ØII.

g. Supercoiled circular DNA was found in each of the R-17 non-sensitive xyl⁺ diploid hybrids which had responded as males when tested with ØII. The sizes of the circular DNA molecules, as determined by sucrose density gradient centrifugation, differed in each hybrid, ranging from a low of 25 million daltons to a high of 150 million daltons. In the 3 R-17 non-sensitive xyl⁺ diploid hybrids which had responded as females when tested with ØII, no circular DNA was found. Negative findings, with respect to the presence of circular DNA, were recorded also for the 3 R-ly non-sensitive xyl⁺ hybrids in which no instability of the xyl⁺ marker was observed (and which had responded as females with ØII) and for WR3026 itself.

h. Segregant cells (xyl⁻) from each of the six xyl⁻ diploid hybrids which responded as males with ØII and contained circular DNA were found, as reported above, to have lost the ability to inhibit ØII plaque formation. When these segregant cells were examined for the presence of circular DNA, none of them was observed to contain it. Thus, the loss of the xyl⁺ marker from each of these hybrids was correlated with loss both of the male response to ØII and of the circular DNA.

i. We examined, for the presence of circular DNA, 3 of the 10 R-17 sensitive, xyl⁺ diploid hybrids which had appeared from the mating experiments, to contain a cytoplasmic F-factor that was not associated with the xyl⁺ marker. Each contained as expected, a supercoiled circular DNA element. The molecular weights of these elements, as determined by sucrose density gradient centrifugation, were in the range of 60 to 70 million daltons, which is reasonably consistent with the 61 million dalton molecular weight of F. When

xyl⁻ segregants from each of these hybrids were examined, they were found to have retained their sensitivity to R-17, as well as their contained, circular DNA elements. It was expected that if the *Salmonella* chromosomal fragment bearing the xyl⁺ marker were a part of the circular DNA element, some change in the size of this element should have been discernible in the xyl⁻ segregants. However, in experiments in which ¹⁴C-labelled, circular DNA obtained from the xyl⁺ version of each hybrid was sedimented in the same sucrose gradient with ³H-labelled circular DNA obtained from its xyl⁻ segregant, no such change was detected. With each of the 3 hybrids tested in this manner, the sedimentation rate of the circular DNA of the xyl⁺ version of the hybrid was indistinguishable from that of the circular DNA obtained from its xyl⁻ segregant. Thus, in these 3 diploid hybrids, we found no indication of a physical association of the contained, F-factor DNA with the unstably conserved *Salmonella* chromosomal fragment.

j. In earlier experiments in which *E. coli* Hfr donors were mated with a recombination deficient (recA) *E. coli* recipient strain the formation of two classes of merodiploid hybrids was observed. The first class consisted of hybrids which behaved as F-prime strains; they exhibited sensitivity to the male-specific phage, MS-2, and transferred at high frequency, in subsequent conjugal unions, the F-associated chromosomal fragments which they had acquired in the initial mating. Hybrids of the second class were resistant to MS-2, and unable to act as donors of the added DNA; nevertheless, they responded as males to the female-specific phage, ØII. In these latter hybrids, the donor-derived genetic fragments were conserved, it was suggested, by association with a portion of F, which allowed replication of the element but which lacked the determinants of F-pili formation and DNA transfer. The interpretation seems the most plausible one also for explaining the male response to ØII exhibited by 6 of the xyl⁺, R-17 non-sensitive diploid hybrids observed in the present study. Likewise, it would account for the conservation of the added *Salmonella* DNA in a circular configuration in these hybrids.

k. In the 5 xyl⁺ mal⁺ diploid hybrids that we encountered, the *Salmonella* DNA seemed to be conserved as part of a functional F-merogenote; in 6 of the xyl⁺, R-17 non-sensitive, diploid hybrids, it was maintained as part of what appeared to be an incomplete or defective F-merogenote. Conservation of the *Salmonella* DNA in the remaining diploid hybrids examined apparently did not involve association with either a complete or an incomplete F. Circular DNA was not found in the 3 R-17 non-sensitive diploid hybrids that responded as females when tested with ØII. In view of the fact that circular DNA was found

in each of the 6 R-17 non-sensitive hybrids that responded as males with ϕ II, the negative findings with the 3 diploid hybrids behaving as females merit a positive interpretation; that is, the Salmonella genetic material contained in them was perpetuated in a manner which did not involve its assuming a supercoiled, circular configuration. Furthermore, in those examined diploid hybrids which behaved as F⁺ strains, the circular DNA contained appeared to represent only the F factor; both genetic and molecular experiments indicated that the added Salmonella chromosomal fragments were not associated with it. The mechanism by which such chromosomal fragments are conserved in partially diploid hybrids in which F is not present or, if present, is not involved with replication of the fragment, has yet to be determined.

3. Hybrids between coliphage λ and Salmonella phage P22

Salmonella strains are normally insensitive to coliphage λ being unable to adsorb this phage due to a lack of appropriate cell-surface receptor (λ r_{cp}). Recent studies, however, have established the feasibility of constructing Salmonella typhosa hybrids, derived from matings with Escherichia coli K-12 Hfr strains, that are sensitive to coliphage λ . Introduction of malB- λ r_{cp} gene cluster from E. coli K-12 into S. typhosa yields hybrids capable of adsorbing λ that are still unable to replicate λ lytically. Addition of further E. coli gene(s) controlling replication of λ (termed λ r_{ep}) to such hybrids results in S. typhosa derivatives that are fully sensitive to λ and that approximate E. coli K-12 in their interaction with λ (2). The λ r_{ep} gene(s) that enable λ to replicate in S. typhosa have been mapped in the 60- to 72-min segment of the E. coli K-12 chromosome. Although not fully resolved, recent observations also indicate the necessity of the E. coli malA cluster for expression of λ sensitivity by S. typhosa hybrids.

b. These findings clearly suggested the possibility of recovering intergeneric hybrids that would facilitate genetic recombination between evolutionary diverse phages, such as coliphage λ and salmonella phage P22. The S. typhosa hybrids shown to be sensitive to λ appear unable to support lytic replication by phage P22. Consequently intergeneric crosses were initiated between E. coli K-12 Hfr donors and a P22-sensitive Salmonella typhimurium recipient to isolate an S. typhimurium hybrid, which in addition to being sensitive to phage P22, also expresses lytic sensitivity to coliphage λ . Such a strain is a suitable host for construction of new hybrid phage species, their genomes being derived from both P22 and λ .

4. Isolation of *S. typhimurium* hybrid with sensitivity to coliphage λ and phage P22

a. Based on previous experience with *S. typhosa* we anticipated that *S. typhimurium* hybrids with lytic sensitivity to λ would have to express at least the malB- λ rcp *E. coli* gene cluster and the xyl- λ linked λ rep *E. coli* gene(s). The possibility that the *E. coli* malA cluster was also essential for expression of λ sensitivity, likewise, had to be considered. Initial attempts to prepare a λ -sensitive derivatives by first crossing with an *E. coli* donor for inheritance of the malB- λ rcp loci and then remating for additional loci were unsuccessful. As a consequence, we performed "double selection" crosses in the hope of recovering a hybrid that, from a single conjugation with an *E. coli* K-12 Hfr, inherited all the necessary *E. coli* determinants for expression of λ sensitivity. *S. typhimurium* WR4026, a Thr-Xyl- derivative of *S. typhimurium* LT7 HfrSC19, was used as a recipient. Although this strain is an Hfr donor, we chose to use it as a recipient, considering it advantageous for future studies to have a λ -sensitive *S. typhimurium* derivative with Hfr donor properties. The donor strain in these initial crosses, *E. coli* K-12 Hfr WR2004, was mated with the *S. typhimurium* WR4026 recipient, and hybrids that had inherited the donor thr⁺ and xyl⁺ markers were isolated on minimal medium containing xylose as the carbon source and lacking threonine. With this double selection system, it was conceivable that some of the hybrids would inherit the malB- λ rcp gene cluster (min. 79) and the λ rcp gene(s) present in the xyl (min. 70) through thr (min. 90) region of the *E. coli* chromosome. Moreover, the close proximity of malA (min. 65) to the xyl marker (min. 70) allowed for its inheritance, since this *E. coli* marker may also be essential for expression of λ sensitivity by *S. typhimurium*. More than 200 such "double selected xyl⁺ thr⁺*S. typhimurium* hybrids were accumulated and subsequently screened for sensitivity to λ . Overnight broth cultures of these clones were spread on TB agar and spotted with drops of a λ cIt1 lysate (1×10^{10} PFU per ml) prepared on *E. coli* K-12. Only one hybrid showed evidence of lysis by λ cIt1, and upon further testing revealed sensitivities to λ vir, λ i434 and sx. When used as an overlay indicator host, this hybrid yielded distinct λ plaques at an efficiency of plating that approximated an *E. coli* K-12 host (0.5-1.0). Subsequent characterization revealed that this clone also inherited the *E. coli* tna⁺ gene region (min. 73) and that the inherited xyl⁺ tna⁺ segment was genetically unstable. When plated on MacConkey xylose medium xyl-tna- segregants were readily detected thus indicating that this hybrid was partially diploid for this chromosomal segment. One such segregant (WR4027), picked at random and found

to conserve sensitivity to λ , was chosen for further study. Additional characterization revealed that WR4027 also expressed E. coli K-12 type 1 pili (pil⁺, min. 89) and the metB allele of the WR2004 E. coli K-12 donor. Moreover, it became immediately evident that WR4027 was not sensitive to phage P22 having become rough in its lipopolysaccharide character. By using the series of "smooth" and "rough" specific phages, P22, 9NA, FO, Ffm, BR60, 6SR, BR2, and C21, we identified the genetic lesion causing the rough lipopolysaccharide property as being a rough A (rfa), core defect. The origin of this rough mutation in WR4027 is obscure. Although S. typhimurium WR4026 appeared smooth and sensitive to P22, reexamination of this stock culture revealed the presence of rfa lipopolysaccharide mutants; thus it was possible that the clone used in our mating with E. coli K-12 Hfr WR2004 was an rfa mutant. Alternatively, the rough lipopolysaccharide character of WR4027 could be a consequence of the mating with E. coli Hfr WR2004, which like other E. coli K-12 strains is rough.

5. Isolation of a P22-sensitive derivative of S. typhimurium WR4027

a. Sensitivity of S. typhimurium to phage P22 depends upon expression of smooth-somatic (O) antigens. The lipopolysaccharide component responsible for O antigen factor 12 is considered to be at least part of the P22 adsorption site. Thus to obtain a P22-sensitive derivative of WR4027, it was necessary to repair the rfa lesion, since this defect in the lipopolysaccharide core prevented attachment of O-specific polysaccharides and led to the P22-resistant, rough phenotype. Our efforts to isolate smooth, P22-sensitive revertants from populations of WR4027 were not successful. We therefore, mated WR4027 (rfa⁻, xyl⁻) with smooth (rfa⁺, xyl⁺) S. typhimurium Hfr WR4016 and selected for inheritance of the donor xyl⁺ marker. Previous studies have shown a close genetic linkage of the xyl and rfa loci. Seven of 25 such hybrids were smooth, being sensitive to phage P22 and resistant to various "rough specific" phages. All seven hybrids retained their sensitivity to coliphage λ . One of these, termed WR4028, was used in our further studies.

b. S. typhimurium hybrid WR4028 as a host for phage P22 and λ . When used as a host for phage P22^{c+} in soft agar titrations, WR4028 yielded P22^{c+} plaques typical of those produced on a native S. typhimurium LT-7 strain. The efficiency of plating of P22 by WR4028 approximated that of the original S. typhimurium parent (efficiency of plating 0.1-1.0). When similar plaque assays were performed with cit1 plaques were faint and often indistinct when low

amounts of phage were plated. Moreover, we observed some variation in plating efficiency and plaque resolution among independent clones of WR4028. In distinct contrast, "rough" S. typhimurium hybrid WR4027, the immediate parent of WR4028, consistently yielded typical distinct λ cItl plaques at a predictable plating efficiency. We consider these variations in the behavior of λ , evident with the "smooth" WR4028 host but not with the "rough" WR4027 host, to be a reflection of an adsorption problem, created by the presence of smooth O-repeat lipopolysaccharide layers. Recent studies on the interaction of λ with S. flexneri indicate that smooth lipopolysaccharide layers interfere with efficient adsorption of λ ; "smooth" host derivatives adsorbed λ poorly when contrasted to "rough" hosts, whose efficiency of adsorption approached that of E. coli K-12.

c. These difficulties with λ plaque resolution on the smooth WR4028 host can be obviated by isolation of host range mutants of capable of producing distinct plaques at a predictable plating efficiency on WR4028. One such derivative, termed λ cItltm, has been isolated by plating λ cItl on WR4028 and picking a plaque that was far more distinct than that typical of λ cItl on this host. After cloning by repeated platings on WR4028, we prepared a lysate of λ cItltm that yielded typical λ plaques on both smooth (WR4029 and rough (WR4027) hybrids.

d. Lysogenization of S. typhimurium WR4028 with P22^{c+} and λ cItl. As expected WR4028 could be readily lysogenized with phage P22^{c+}. A high proportion of bacterial clones isolated from plaques of P22^{c+} were P22 lysogenic derivatives of WR4028. They expressed an immunity to P22_{c2}, and spontaneously or upon induction with mitomycin C, produced free P22^{c+}.

e. S. typhimurium WR4028 was readily lysogenized with coliphage λ . Drops of λ cItl lysate, prepared on E. coli K-12 were deposited on lawns of WR4028, and after overnight incubation, colonies from the area of lysis were purified and scored for their lysogeny. All eight clones so tested were lysogenic for λ cItl, expressing immunity to λ cItl and sensitivity to λ imm434 and λ vir, and produced free λ cItl phages. One such derivative chosen for further examination responded to temperature induction of λ cItl in a manner similar to an E. coli K-12 (λ cItl) lysogen. To exclude the possibility that this derivative was lysogenized with a host range λ cItltm mutant rather than λ cItl, we assayed this induced lysate on E. coli K-12 and S. typhimurium WR4027 (rough) and WR4028 (smooth). The plaque morphology on E. coli K-12 and WR4027 was typical of λ cItl. On the smooth WR4028 host, the plaques were very faint and indistinct, paralleling our previous findings with λ cItl. This strain was next lysogenized for P22^{c+} yielding a derivative lysogenic for both λ cItl and P22^{c+}. The feasibility of preparing various lysogens of S.

typhimurium WR4028, needed for formation of hybrid phage species, was thus established by these experiments.

6. Isolation of new phage species

a. The dual sensitivity of S. typhimurium hybrid WR4028 to phage P22 and λ and the availability of various lysogenic derivatives of this strain enabled us to isolate hybrid phages between these two phages. Phage P22 was grown on various λ lysogens of S. typhimurium WR4028, and about 10^9 PFU of such lysates were plated on a λ lysogenic derivative of WR4027 (rough), which was immune to λ and resistant to phage P22. After overnight incubation at 37° , about 50 small plaques were detected. Several of these were cloned and tested to determine whether they were antigenically identical to coliphage λ . Antiserum against coliphage λ , which inactivated λ at a rate constant, k , of 156 min^{-1} , neutralized the new phage isolates with a rate constant of $148 \pm 3 \text{ min}^{-1}$. No inactivation of these phages was detected with antiserum against P22 ($k = 2200 \text{ min}^{-1}$). These data indicated that the tail antigens responsible for adsorption of this new phage isolate were serologically identical to those of λ . Due to their antigenic structure and capacity to plate on a P22-resistant, λ lysogen of S. typhimurium, we considered these clones to represent hybrids between λ and P22, henceforth designated to the λ -P22 class. The λ -P22 class forms plaques on λ lysogens of both WR4028 and WR4027. Although λ -P22 plaques appear small, it was evident that the c markers of λ -P22 plaques mimic those of the P22 strains used in preparation of lysates of λ lysogens, regardless of the λ c marker present. For example, when a wild-type P22 \bar{c}^+ strain is used for infection, the λ -P22 hybrids exhibit turbid plaques. In contrast, infection with the c1, c2, or c3 clear plaque mutants of P22 yields a λ -P22 derivatives that express the corresponding degrees of clearness typical of P22 mutant used. In addition, when a λ -P22 \bar{c}^+ hybrid class contains at least the c locus of phage P22 and conserves the protein of coat of λ .

b. We next attempted to isolate hybrid phages exhibiting the P22 protein coat with the c region of λ . Efforts to isolate this class (termed P22- λ) by plating on a P22 lysogen of a λ -resistant derivative of WR4028 were not successful. We could infrequently detect this class of hybrids, however, by examining for distinct superimposed plaques on confluent lysis plates of a λ lysogen of WR4028 infected with P22 stocks previously propagated on this lysogen. After purification by plating on a λ -resistant derivative of WR4028, we found that the P22- λ hybrid class expresses the P22 protein coat and the c marker of the P22 strain used. This conclusion was based on our finding that antiserum against P22 inactivated P22 and representa-

tives of the P22- λ class at about the same rate constant ($k = 2200 \pm 10^{-1}$). No cross neutralization was detected with anti-serum against λ ($k = 155 \text{ min}^{-1}$). In addition, backcrosses of a P22- λ c^+ hybrid with λcII yielded λc^+ derivatives at a frequency of about 0.13%.

c. Derivatives of WR4028, lysogenized with either a λ -P22 or a P22- λ hybrid were scored for their pattern of immunity to parental and hybrid phages. λ -P22 hybrids form plaques on λ lysogens of both WR4027 and WR4028, but not on P22 lysogens of WR4028. Moreover, λ -P22 lysogens of *S. typhimurium* WR4028 are sensitive to λ because this phage hybrid contains the P22 c region in place of the λc region. The λ -P22 lysogen tested, however, was sensitive to P22, indicating inheritance by the phage of the c region of P22, but not of the P22 Im region essential for conferring immunity to P22. In contrast to P22, only the genes in or near the c region of λ function in control of immunity. Our findings of an absence of P22 Im locus in the λ -P22 hybrid is analogous to the observation that the P22 Im region is retained by the P22- λ hybrid. The P22 lysogen of WR4028 shows immunity to the P22- λ hybrid used. In addition, the P22- λ lysogen of WR4028 is immune to both P22 and λ . It thus appears that the P22- λ hybrid tested possesses the Im region of P22 while inheriting the c region of λ .

Summary and Conclusions

1. *Salmonella typhimurium* hybrids expressing the *S. typhosa* antigens 9, d, and Vi were constructed by genetic crosses with an *S. typhosa* Hfr donor. The hybrids retained the same degree of mouse virulence as their *S. typhimurium* parent strain, the minimum lethal dose being less than 50 organisms when tested either in C₅₇ black mice or Swiss white mice. Vaccination of the Swiss white mice with *S. typhosa* Ty2 vaccines prepared by acetone treatment, alcohol treatment, or heat-killing conferred significant protection against challenge by the hybrid strains but not against their *S. typhimurium* parent. Both the acetone-treated and alcohol-treated typhoid vaccines were markedly more protective than the heat-killed, phenol-preserved vaccine.

2. Partially diploid *Escherichia coli* K-12 hybrids recovered from mating with a *Salmonella typhimurium* Hfr strain were found to differ with respect to the manner in which they conserved the added *Salmonella* deoxyribonucleic acid (DNA). Five of the diploid hybrids examined appeared to maintain the *Salmonella* DNA as part of a functional F-merogenote; these hybrids were sensitive to the male-specific phage, R-17, responded as males to the female specific phage, ϕ II, and transferred their inherited *Salmonella* genetic markers at high

frequency in conjugation experiments. Six diploid hybrids were observed which were not sensitive to R-17, and from which the added Salmonella DNA was not transmissible in conjugation tests; nevertheless, these hybrids responded as males to ϕ II, and the Salmonella chromosomal fragments were conserved in them as parts of supercoiled, circular DNA elements. It was concluded that these circular DNA elements were defective F-merogenotes, unable to direct the synthesis of F-pili. Three diploid hybrids were found which were not sensitive to R-17, and which responded as females to ϕ II; no circular DNA was found in them, and it was concluded that their conservation of the Salmonella genetic fragments was accomplished in some manner which did not involve association with F or assumption of the supercoiled circular configuration. Other partially diploid hybrids were observed which appeared similar to these latter three hybrids with regard to their conservation of the Salmonella DNA, but which also contained an infecting F-factor; in these hybrids, both genetic and molecular experiments indicated that the unstably conserved Salmonella DNA was not associated physically with the F-factor.

3. An unusual Salmonella typhimurium hybrid with sensitivity to coliphage λ and salmonella phage P22 has been recovered from matings between an Escherichia coli K-12 Hfr donor and an S. typhimurium recipient. The hybrid is an excellent host for achieving genetic recombination between λ and P22. Two broad classes of hybrid phages were isolated. The λ -P22 hybrid class which has the protein coat of λ , contains at least the c region of P22. The P22- λ hybrid class has the protein coat of P22 and has inherited at least the c marker of λ .

Project 3A062110A822 MILITARY INTERNAL MEDICINE

Task 00, Military Internal Medicine

Work Unit 122, Microbial Genetics and taxonomy

Literature Cited.

Publications.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION ^a	2. DATE OF SUMMARY ^a	REPORT CONTROL SYMBOL DD-DR&E(AR)636	
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23(U) To define histopathologic manifestations of injuries and diseases which have current or potential problems in military personnel. The current effort is directed toward studies of the digestive tract and kidney with infections and injuries. These studies provide a basis for a comprehension of pathogenesis, prognosis and treatment.							
24(U) Various morphologic techniques including histology, histo- and cytochemistry, autoradiography, immunofluorescent microscopy, transmission and scanning electron microscopy are employed.							
25(U) 72-07-73 06 EM studies have determined ultrastructural characteristics of spiral microbes which are unculturable fastidious anaerobes, yet common in the normal flora in the digestive tract of man and monkeys. Studies of experimental E histolytica infections indicates that penetration of the gut epithelium by amoeba occurs without producing histolysis and results from the secondary involvement of altered microcirculation. Experimental ulcer formations were investigated in the duodenum of the guinea pig due to fasting, and rat stomachs under stress; the former showed depletion of mucus of Brunner's glands and altered kinetics of epithelial renewal while in the latter altered microcirculation played a major role. Immunopathologic study of kidney lesions associated with experimental trypanosomiasis in monkeys indicated the renal lesion represented membrano-proliferative glomerulonephritis with immune deposits consisting of C3, properdin, occasional IgM, while serum C3 was significantly reduced. Experimental obstructive nephropathy in rabbits have provided evidence that interstitial fibroblasts were capable of acquiring characteristics of smooth muscle cells.							
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Project 3A062110A812 MILITARY INTERNAL MEDICINE

Task 00 Military Internal Medicine

Work Unit 123 Histopathologic Manifestations of Military Diseases
and Injuries

Investigators.

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Description

To define histopathologic manifestations of injuries experimentally produced and diseases which present current or potential problems in military personnel. The current effort is directed toward studies of diseases of the digestive tract and kidney due to infections and injuries. These studies provide a basis for a comprehension of pathogenesis, scientific treatment, and determination of prognosis in diseases and injuries in military personnel.

Approach to the Problem

A multi-disciplinary approach including conventional histology, histo- and cytochemistry, autoradiography, immunofluorescent microscopy, transmission and scanning electron microscopy is employed.

Progress

In the past, this work unit was primarily concerned with studies of histopathologic manifestations of acute diarrheal diseases of infectious origin. In the past two years, we have expanded this work unit and included studies of diseases of the digestive tract of non-infectious origin and experimental infections and injuries in the kidneys.

I. STUDIES OF HOST-INDIGENEOUS MICROBE RELATIONSHIP IN THE COLONIC MUCOSA.

Medical microbiology has been concerned primarily with the potentially pathogenic members of indigenous microflora. Symbiotic species are of at least equal importance because they maintain essential anatomical and physiological function with the host (Dubos 1967). The lumen of the

digestive tract is now acknowledged as the site of a dynamic ecological system composed of extremely large populations of different microbes maintained in balanced proportions.

Studies have indicated that indigenous microbes are not randomly present in the lumen of the gut but populate definite anatomical and histologic divisions of the gastrointestinal tract (Savage, Dubos & Schaedler 1968). A recent study indicated that the large concentration of epithelial-associated indigenous microbes resist access of pathogenic bacteria to the intestinal epithelial surface (Savage 1972).

Little is known about "intestinal epithelial cell-associated indigenous microbes" in mammals including monkey and man (Nelson & Mata 1970). For this reason, we have expanded our previous study on spiral-shaped microbes in the colonic mucosa of the rhesus monkey and man (Takeuchi & Sprinz 1969) and rat.

A. Studies of Spiral-shaped Microbes in the Colonic Mucosa of the Rhesus Monkey

Spiral-shaped microbes are one of the most common components of the indigenous flora of the large bowel in mammals including monkeys and man. They are fastidious anaerobes and, therefore, difficult or impossible to culture and isolate. Little is known of their structural characteristics and their relation to the host. Our earlier studies on spiral-shaped organisms which characteristically colonize in almost pure culture on the surface of the colonic epithelium of healthy monkeys and man have been expanded. In the rhesus monkey, we have established for the first time the structural characteristics of this fastidious anaerobic spiral-shaped organism and its relationship to the host-cell.

1. Host Cell Relationship: Previous studies from this department indicate that about 12% of normal rhesus monkeys and 3% of normal human subjects have spiral-shaped microbe infestations on the colonic mucosa which are indistinguishable in both hosts (Takeuchi & Sprinz 1970).

Utilizing conventional histology, transmission electron microscopy (TEM), and scanning electron microscopy (SEM), we investigated spiral microbe-epithelial cell relationship in the colon of the healthy rhesus monkey.

In paraffin sections stained with H&E, the brush border of the normal colonic epithelium was narrow and uniformly stained by eosin (Fig. 1). However, the brush border of the colonic epithelium infested by spiral organisms was broader than normal and characteristically basophilic (Fig. 2). The Warthin-Starry stain, a silver stain for spirochetes, intensely colored the infested brush border. The Gram staining was inconsistent.

Spiral-shaped organisms appeared to be confined to the brush border and not to penetrate deeper. Only surface epithelial cells including crypt openings, were infested; the depth of the crypt was not infested. There were no structural changes in the infested mucosa, and the lamina propria exhibited no inflammatory response.

At less than 100X magnification, photographs of the surface of the normal colonic mucosa obtained by SEM were fairly comparable to those obtained by the dissecting light microscope. At 100X magnification under SEM, the colonic mucosa with spiral-shaped microbe infestation was similar to the normal counterpart; the surface was regularly arranged in a geometrical pattern consisting of numerous polygonal units (Fig. 3 & 4). At magnification from 100X to 300X, the surface of the normal mucosa appeared finely granular. In contrast to the normal mucosa (Fig. 3), in the infested mucosa the polygonal units were circumscribed by less well-defined furrows (Fig. 4). The polygonal units and the central holes (crypt orifice) were better seen at magnifications of up to 1000X; the ridges observed on the normal mucosa (Fig. 5) under SEM at a comparable magnification were absent in the infested mucosa (Fig. 6). They were completely replaced by a coarsely granular surface, which corresponded to the spiral-microbe infested brush border, seen as hairy fuzz in light microscopic preparations (Fig. 2). The furrows outlining polygonal units were occasionally obscured by the presence of multiple spirochetes at the surface.

At a 3000X magnification under SEM, the coarsely granular surface was further resolved into multiple closely packed spirochetes oriented perpendicular to the mucosal surface. Numerous organisms extended into the crypt through the crypt orifice (Fig. 6).

At magnifications of 10,000X under SEM, spirochetes could be well-distinguished as an individual organism and were often attached to each other especially at their lower one third (Fig. 7-8). They were approximately up to 5nm and 0.5nm in diameter. Quantitative counts were made on the number of spirochetes at a photographic

magnification of 30,000X; the approximate figure for the density of spirochetes at the surface was estimated to be 1,700 organisms per square millimeter.

At higher magnification under TEM, individual, slender, gently curved, spiral-shaped organisms were seen (Fig. 9), which measured up to 6nm in length and 0.5nm in diameter. At even higher magnification, they were identified as two structurally different microbes - a flagellated microbe and a spirochete.

Spirochetes and flagellates were the only two organisms identified in the brush border. The ratio of the two types of organisms varied from one case to another but spirochetes always predominated.

The brush border of normal colonic epithelium, which is composed of four distinct ultrastructural components, was heavily penetrated by both organisms as illustrated in Fig. 9. The glycocalyx, the most superficial component, was absent. The microvilli, located beneath the glycocalyx, were almost completely destroyed; the few remaining were blunted and had irregular contours. The next deeper component, the terminal web, was attenuated and was abutted by the penetrating portions of the organisms. The deepest component, apical cytoplasm, remained unaltered. Embedded side by side, both types of organisms were always arranged perpendicular to the brush border where they closely abutted the invaginated plasmalemma of the epithelial cell (Fig. 9,10). Sometimes, the coiled flagella of the flagellate assumed an advanced position in front of the organism against the host cell plasma membrane (Fig. 11). More often, however, the flagella turned backwards from the tip of the organism and coiled around it.

2. Ultrastructural Identification of Spirochetes and Flagellated Microbe: In order to determine ultrastructural characteristics of two different spiral organisms, we used the following three techniques of electron microscopy - conventional thin-sectioning, negative contrast and metal shadowing techniques.

Results - The structural character of spiral-microbes determined by these three techniques are summarized in Table 1.

Spirochetes had 2-6 spirals, gently tapered ends, and measured from 3-6 μ m in length and 0.2-0.4 μ m in diameter. This organism had a central protoplasmic cylinder (cytoplasm) enclosed by a cytoplasmic membrane which appeared as a single dense layer, 6.0-8.0nm, and an outer external coat (cell wall). The external coat appeared as a triple-layered structure 14-16nm thick (Fig. 9,10), consisting of two dense layers. Between the cytoplasmic membrane and the cell wall was

TABLE I
STRUCTURAL CHARACTER OF SPIROCHETE AND FLAGELLATE

Ultrastructural Characteristics	Spirochete	Flagellate
Length of organism	3-6 nm	4-6 nm
Width of organism	0.2-0.4 nm	0.2-0.4 nm
Number of spirals	2-6	2-4
End contour	tapered	blunt
Fibrils	20 nm in diameter 4 or 6 at each end originating from terminal disc	none
Flagella	none	1-4 μ m in length and 3.5 + 2.5 nm in diameter; originating from flagellar stub
Cell wall thickness	14-16 nm	10 nm
Space between cell wall and cytoplasmic membrane	30-100 nm contains fibrils	20 nm homogeneous dense matrix
Cytoplasmic structures		
ribosomes	present	not visualized
mesosomes	present	not visualized
round electron-lucent punched-out areas	absent	present

a moderately dense homogeneous space which was occupied in part by a group of fibrils located at one side.

Some spirochetes showed 12 individual fibrils at the midportion and 6 fibrils at each end (Fig. 12 & 13); others revealed 8 fibrils at the midportion and 4 fibrils at each end (6-16-6 and 4-8-4 arrangement). All axial fibrils wound around the protoplasmic cylinder in a helical form and extended from one end of the organism to the other (Fig. 13). The fibrils, originating from opposite ends, overlapped, thus giving the impression, when viewed only in the area of overlap, that the spirochete had twice as many fibrils. At each end, the fibril made a sharp bend before insertion into a disc-like structure (Fig. 12), which measured approximately 40nm in diameter. This disc-like structure extended through the entire length of the organism and corresponds to the "annular swellings" seen in Reiter treponema (Ryter & Pillot) and the "terminal disc" seen in Spirochete aurantia (Breznak and Canale-Parola) and oral spirochetes (Listgarten & Socransky) (Fig. 13). The cytoplasmic cylinder contained uniformly distributed ribosomes and ill-defined areas of electronopacity containing fine meshes of dense strands. These ill-defined areas appeared to be the mesosome of the organism. The characteristic number of the axial fibrils, 8-12-8, the size of the cytoplasmic cylinder, and the structure of the outer envelope were identical to those of Borellia vincentii studied by Listgarten & Socransky (1964 & 1965).

The flagellated microbe was seen less frequently than spirochetes. It had 2-4 spirals and ranged from 4-6nm in length and 0.2-0.4nm in diameter; therefore, size alone could not distinguish these two organisms. The ends of this latter organism were distinctly different in that they terminated bluntly, and a single flagellum emanated from each end, which identified it as a flagellate. The flagellate was composed of a protoplasmic cylinder, cell wall, and polar flagellum at each end. The cell wall, which was approximately 10nm thick, consisted of a composite of two electron-dense layers 3.0nm in thickness, separated by a less electron dense homogeneous layer of 4.0nm. There was a moderately dense homogeneous layer, approximately 20nm, between the cell wall and the cytoplasmic membrane. Occasional ill-defined opaque masses appeared in the cytoplasmic cylinder. Flagella varied from 1-6µm in length and 3.5 ± 2.5 nm in diameter and originated at each end of the cytoplasmic cylinder from a "flagellar stub" (Fig. 11, 14, 15 & 16). The flagellum consisted of a sheath and core; the sheath measured 6.0 ± 1.5 nm, and the core, 14.5 ± 1.5 nm in diameter. The structure of the flagella and flagellar stub were similar to that of flagellated bacteria such as Vibrio fetus and Proteus vulgaris.

Discussion

In the past, spiral-shaped microbes recovered from the gastrointestinal tract or stools of mammals were given different names such as spirillum, leptospirillum, spirochetes, vibrio, and bacterioid (12). Identification in many instances was based mainly on morphological characteristics in tissues and direct smears observed by light microscopy. As exemplified in this study, the light microscope cannot adequately identify spiral-shaped microbes. The use of a single technique in electron microscopy is also inadequate. SEM is capable of demonstrating the natural environment of spiral microbes; however, it is incapable of resolving their relationship with the host and their structural characteristics. Thin-sectioning technique provides a view of the host-microbe relationship but does not give complete information about the microbe. Negative-staining technique does give more information about the microbe structure, but does not provide a view of the host-microbe relationship. Each technique provides conclusive information but has distinctive limitations, however, collectively these techniques give a more accurate picture of host-parasite relationship.

Prior to the advent of SEM, the dissecting microscope proved indispensable in illustrating changes of the mucosal surface architecture of the small and large intestine, but offered limited resolution. The three-dimensional reconstruction of intestinal biopsies from serial sections proved useful in demonstrating architecture of the gut mucosa but was cumbersome. With superior scanning capabilities and great depth of focus, SEM, at higher magnification, can provide a far clearer image of the surface structure. With this new tool, the present study has clearly shown how intestinal spirochetes inhabit their natural environment. Three-dimensional views reveal that organisms characteristically flourish almost as a pure culture on the mucosal surface of the colonic mucosa.

With SEM observation, we were able to demonstrate the spatial relationship between spiral microbes and the colonic mucosa. No organisms were randomly oriented on the mucosal surface; all were well-oriented perpendicularly to the surface. This spatial relationship suggests that organisms may utilize their forward portion for nutritional intake from the host cells. Consequently, this unique orientation may permit equal access for each organism to the host cells, while simultaneously providing such access to a maximum number of spirochetes.

SEM was capable of counting the number of spiral microbes on the mucosal surface of the bowel. This method of counting directly bacterial flora has advantages: (a) The problem of bacterial viability and retrievability is bypassed, and (b) The variation in bacterial population, which is averaged by dilution counting techniques, can be appreciated. However, this method of counting makes no distinction between viable and nonviable organisms. The combination of dilution counting and SEM counting was not possible in this study, since these spiral organisms are not culturable.

Spiral-shaped microbes exclusively and intimately populated the brush border. This unique host-microbial relationship was further clarified by the thin-sectioning technique; spiral organisms do invade host cells. Yet, this invasion was always limited to the upper three cytocomponents, glycocalyx, microvilli, and terminal web. Despite these remarkable alterations, the remaining host cellular structure was unchanged. This implied that cell structures may be selectively destroyed without apparent ill effect. No attempt was made in this study, however, to detect specific functional alterations.

The same techniques have been used to describe spirochetes and spiral-shaped flagellates in the colon of mice by Savage et al and by Davis et al. These organisms are located in the lumen, often enmeshed in mucus, intermixed with fusiform bacteria and sometimes are close to the brush border. They never reside within or cause structural alteration to the brush border. Since some of the luminal spiral-shaped organisms described in mice and rats are ultrastructurally similar to the ones demonstrated in this report, their location and host-cell relationship become important criteria in distinguishing between spiral-shaped organisms in different hosts. In particular, the intimate cohabitation of spirochetes and spiral-shaped flagellates in the brush border of the colonic epithelium of monkey and man represents a unique host-microbe relationship.

Conclusion

Intestinal spirochetes share the general ultrastructural characteristics of other types of spirochetes. The variable number of axial fibrils (4-8-4 or 6-12-6) in other spirochetes has been discussed elsewhere (Listgarten 1966). The intestinal spirochete bearing the axial fibrils of 4-8-4 arrangement is indistinguishable from *Borrelia vincentii*, grown in pleuropneumonia-like organism media in 95%O₂ and 5%CO₂, reported by Listgarten & Socransky. At present, it cannot be concluded from an ultrastructural description alone that both oral and intestinal spirochetes are identical organisms.

The traditional methods of microbiological identification of bacteria--in vitro culture, isolation and identification--are not applicable to spiral-shaped anaerobes which are difficult or impossible to culture. When cultured, infrequently as that may be, the structure of spirochetes can be altered by the artificial environment (Listgarten 1964). In contrast, in vivo ultrastructural identification of spiral-shaped organisms by-passes the problem of culture and provides a detailed description of the structure of the organism and its host relationship. In vivo ultrastructural techniques may provide a new approach to the recognition and classification of spiral-shaped organisms in the digestive tract.

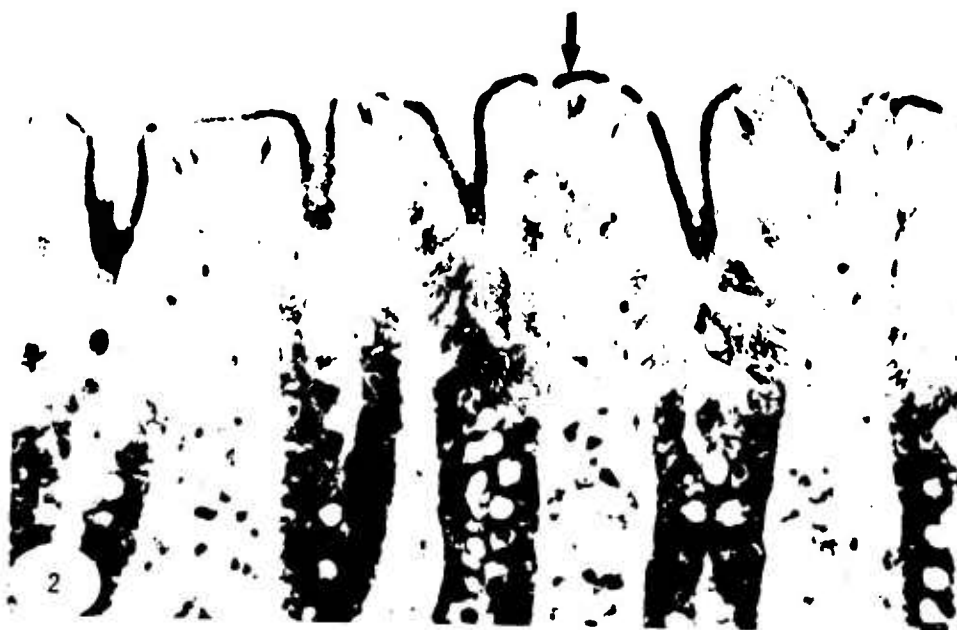
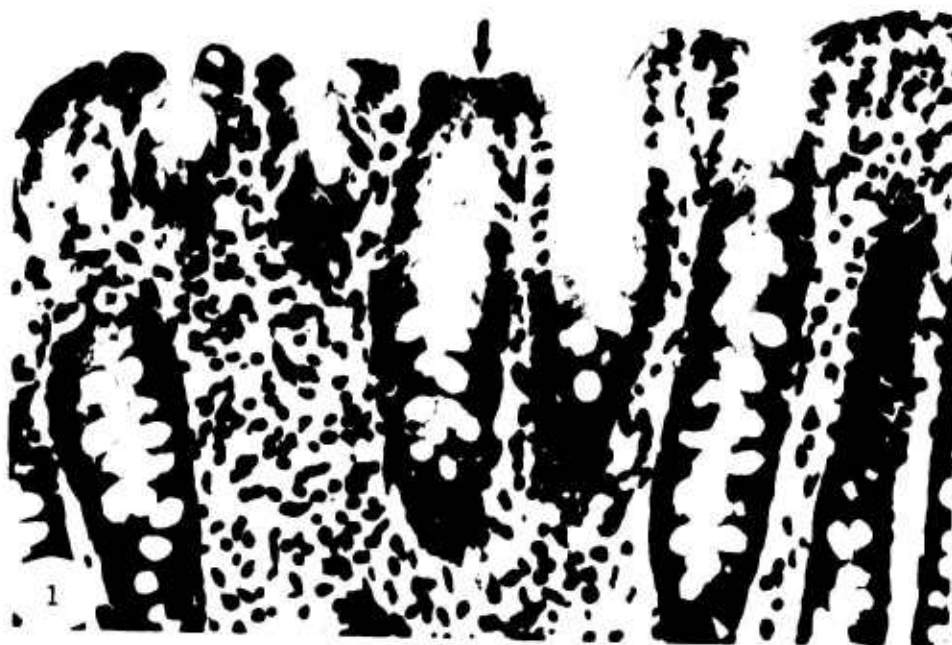


Fig. 1 - Superficial colonic mucosa, normal rhesus monkey. A thin, regular brush border (arrow) is present on the surface epithelium. Hematoxylin and eosin.X380.
 Fig. 2 - Superficial colonic mucosa infested with spiral-shaped organisms, rhesus monkey. A thick haze at the brush border region (arrow) depicts the multiple spirochetes and flagellated microbes. X380.



Fig. 3 - SEM view. The normal surface is divided into polygonal units defined by furrows (dotted lines). The polygonal unit shows a central hole (arrows). X300.

Fig. 4 - SEM view of spirochete-infested mucosal surface. The finely granular surface is different from the convoluted ridges of the normal control (Fig. 3) crypt orifice (arrows). X300.

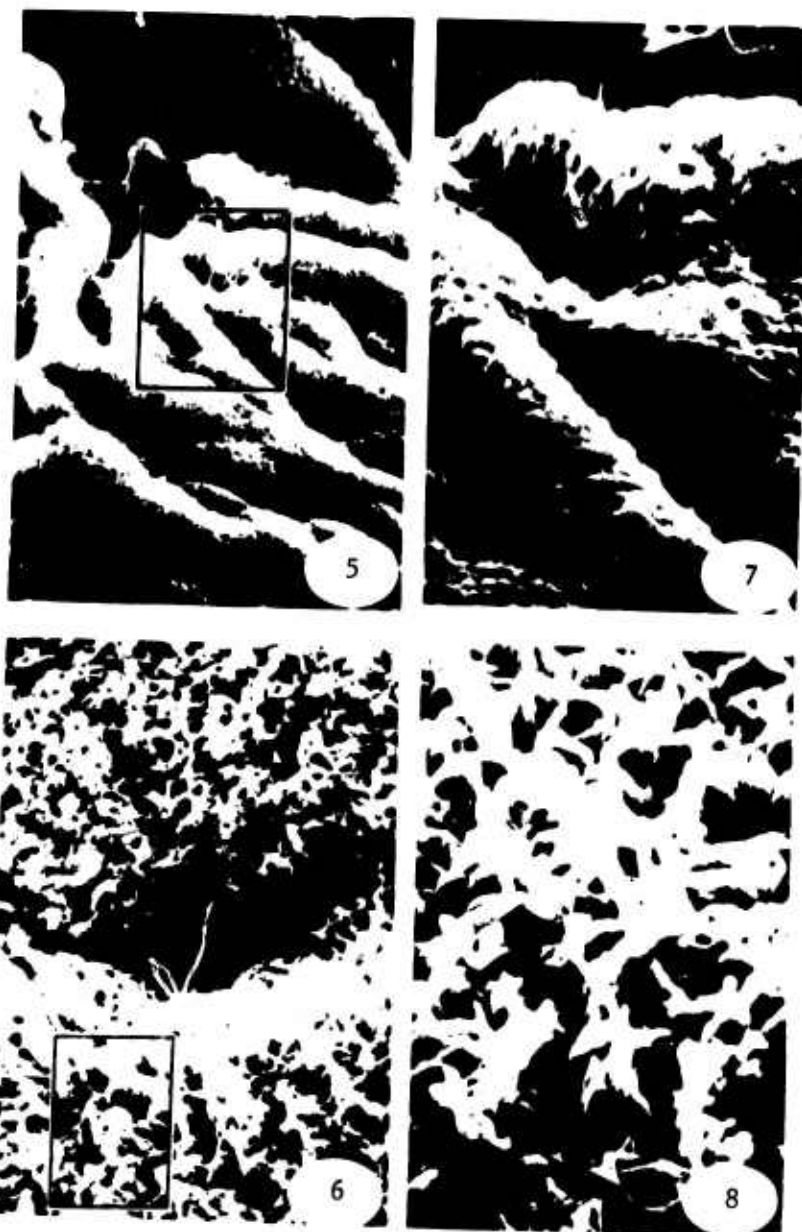


Fig. 5 - SEM view, mucosal surface of normal colon. X3000.

Fig. 6 - SEM view, spirochete-infested colonic mucosa. The normal mucosal surface is completely replaced by numerous spirochetes. The central hole is a crypt opening. X2000.

Fig. 7 - SEM view, mucosal surface of normal colon. Semi-spherical elevations average 3nm in diameter and 2nm in height. X10,000.

Fig. 8 - Higher magnification of SEM view illustrates the individual spirochetes. Organisms are often attached to each other. X10,000.



Fig. 9 - TEM illustrates the spirochete-infested brush border region of an epithelial cell. Numerous spirochetes averaging 5μ in length and 0.5μ in diameter almost totally replace microvilli (MV). The spirochetes consists of cytoplasm enclosed by a cytoplasmic membrane (small arrow) and an outer cell wall (large arrow). There are characteristic axial fibrils (semicircular lines). X48,000.

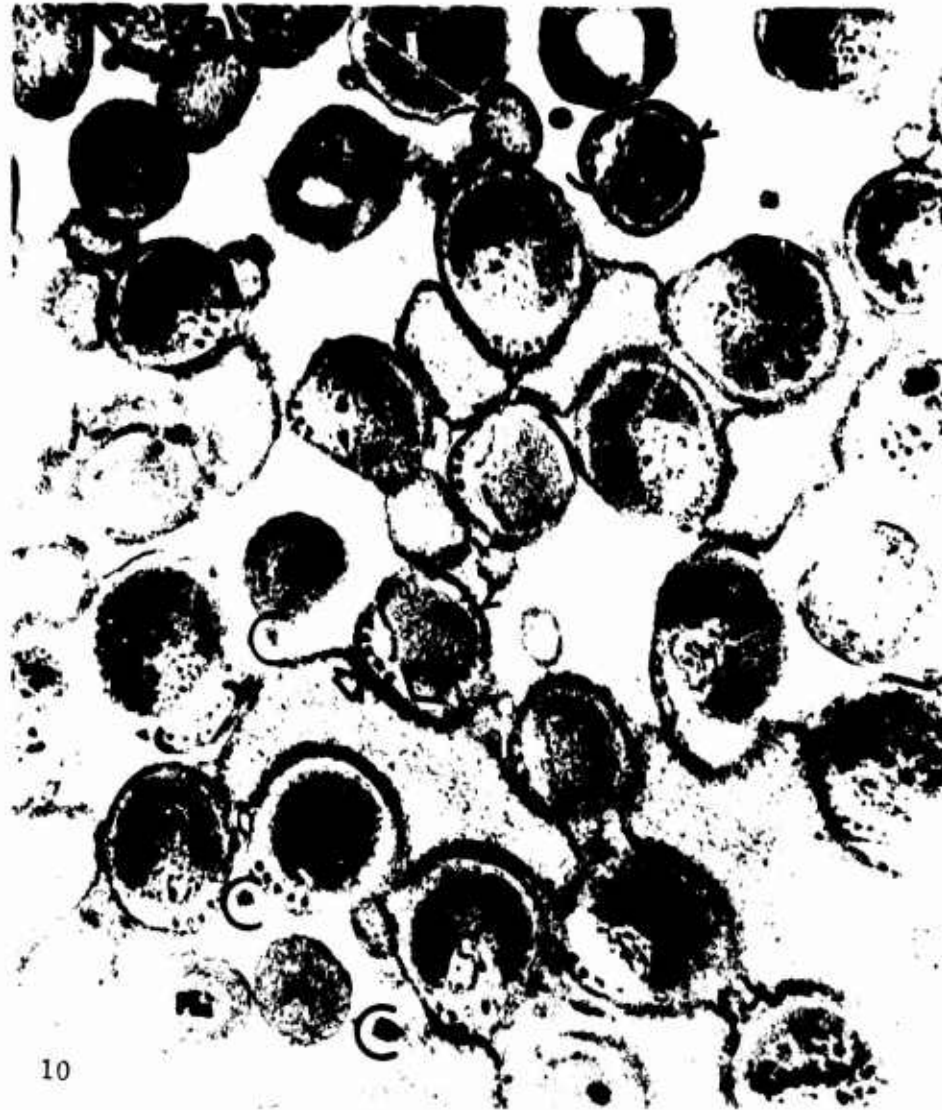


Fig. 10 - Cross-section at the brush border infested predominantly by spirochetes. Spirochetes show several individual axial fibrils (parenthesis) which are located between the cell wall (large block arrow point) and the cytoplasmic membrane (small black arrow point). Flagellates (FL) show absence of axial fibrils. Portions of the flagellum (semicircular line) are seen in proximity to flagellates. Note that the cell wall (large arrow) of spirochetes abuts the plasmalemma of the brush border cytoplasm (small arrow). X98,000.



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Fig. 11 - Terminal web region infested by flagellated microbes. In upper right corner, a flagellum originates from the blunted end of the organism (large black arrow) and coils around itself and indents the host cell plasmalemma. In the upper left corner, a flagellum, at cross section (small arrows), similarly indents the host cell membrane. The cell wall (CW) of flagellates is closely approximated to the trilaminar unit membrane (OL: Outer leaflet; IL: Inter leaflet) of the host cell. A spirochetes (SP) characterized by abundant ribosomes is close to adjacent flagellates. X119,000.



Fig. 12 - Terminal bends in axial fibrils lead to attachment discs (arrows) in protoplasmic cylinder of the spirochete. Negative contrast. X135,000.

Fig. 13 - Spirochete shows six terminal discs at each end from which individual fibrils originate, extend, and wind around the protoplasmic cylinder like a helix. Negative contrast. X36,000.

Fig. 14 - Flagellate shows bipolar flagellation and punched-out round areas in the cytoplasm. Negative contrast. X32,000.



Fig. 15 - Flagellated microbe shown by the shadow-casting technique. A long flagellum originates from one end of the organism. X42,000.

Fig. 16 - A polar flagellum originates at one end of the cytoplasm (arrow) and extends through the cell wall. Negative contrast. X47,000.

B. Studies of Spiral-Shaped Microbes in the Rat Cecum.

This study was attempted to determine whether any specific bacterial population is localized in the cecum of the normal rat, a common experimental animal. Fifteen young male Wistar rats were fed D&G laboratory diet and allowed tap water ad libitum. They were anesthetized and perfused through the heart with chilled fixative.

The following procedures were conducted: 1. Cecal contents were gently scraped from the mucosa, then homogenized in the cold fixative. The homogenized material was filtered through gauze to remove most coarse cellular debris. The filtrate was prepared for the following techniques: examination by phase contrast microscopy; Gram, crystal violet and iodine staining; negative staining for TEM. 2. Portions of cecum were processed for conventional histology and thin-sectioning technique for TEM.

All rats examined showed that the crypts were packed with organisms whose structural character differed from those of the cecal lumen. Organisms found in the cecal lumen consisted of gram-positive and gram-negative rods, cocci and also spiral organisms. In contrast, almost every crypt was populated solely by spiral organisms.

By thin-sectioning and negative contrast technique, spiral-shaped organisms were divided into three types according to their ultrastructural characteristics; true spirochetes, flagellated microbes and microbes with helically coiled fibers. The details of these structural characteristics are being studied. Individual crypts often exhibited a majority of one of the three types of organisms, whereas many other crypts in the same rat showed an intermingling of two or three types of organisms. Occasionally, spiral-shaped organisms were seen within mucus granules of goblet cells and also in the lamina propria, eliciting inflammatory response.

In this study we have noted that in the normal adult rat cecum, spiral-shaped organisms are closely associated with the cecal mucosa and occasionally penetrate it with no noticeable effect on the host. These observations lead to a few intriguing questions. If the host is associated with the normal flora from infancy, is it tolerant for some members of the normal flora especially with regard to flora that may penetrate the mucosa? Would such tolerance enable certain organisms to resist the host's defenses if they share antigenic determinants with the host's normal flora? Would alterations in the normal flora be beneficial or harmful to the host during certain infections? To our knowledge, no data in the literature can satisfactorily answer any of these questions. Further studies on spiral-shaped microbes in vivo may provide some clue in answering these questions.

II. STUDIES ON HOST-PATHOGENIC MICROBES IN THE GUT MUCOSA

A. Studies on the Gut Mucosa Challenged with *Shigella dysenteriae*.

In collaborative studies on *Shigella dysenteriae* infections in experimental animals and human volunteers, we have described histopathologic changes in the colonic mucosa infected by this classic strain of shigella. (See Annual Report, 1972, Department of Applied Immunology, DCD&I, WRAIR).

B. Study on the Small Intestinal Mucosa of the Rabbit Loop Inoculated by *Shigella dysenteriae*-derived Enterotoxin.

Shigella dysenteriae 1 enterotoxin inoculated into ligated ileal loops of rabbits produced alterations and extrusion of villous epithelial cells as early as 1 hour after challenge. By 6 hours, epithelial cells were cuboidal rather than columnar; the villi were shortened with a decreased villus-to-crypt ratio and with many intact or degenerating transmigrating lymphocytes. Neither *Vibrio cholerae* enterotoxin nor heat-inactivated *Shigella* toxin caused any detectable alterations in the mucosal architecture. These data indicate that *S. dysenteriae* 1 enterotoxin is cytotoxic to the intestinal epithelial cell in vivo. The changes induced are similar to those previously found after oral challenge of guinea pigs with live, virulent *Shigella flexneri* 2a. These results suggest that some of the virulence of *Shigella* species in the gastrointestinal tract may be mediated by cell-free toxins. They further serve to distinguish the mode of action of *Shigella* enterotoxin from that of cholera enterotoxin.

III. STUDIES OF EXPERIMENTAL ULCERS IN THE DIGESTIVE TRACT

Pathogenesis of experimental ulcer formation was studied in guinea pigs and rats: the former was produced in the duodenum by fasting and the latter in the stomach by stress.

A. Acute Duodenal Ulceration in the Guinea Pig due to Fasting.

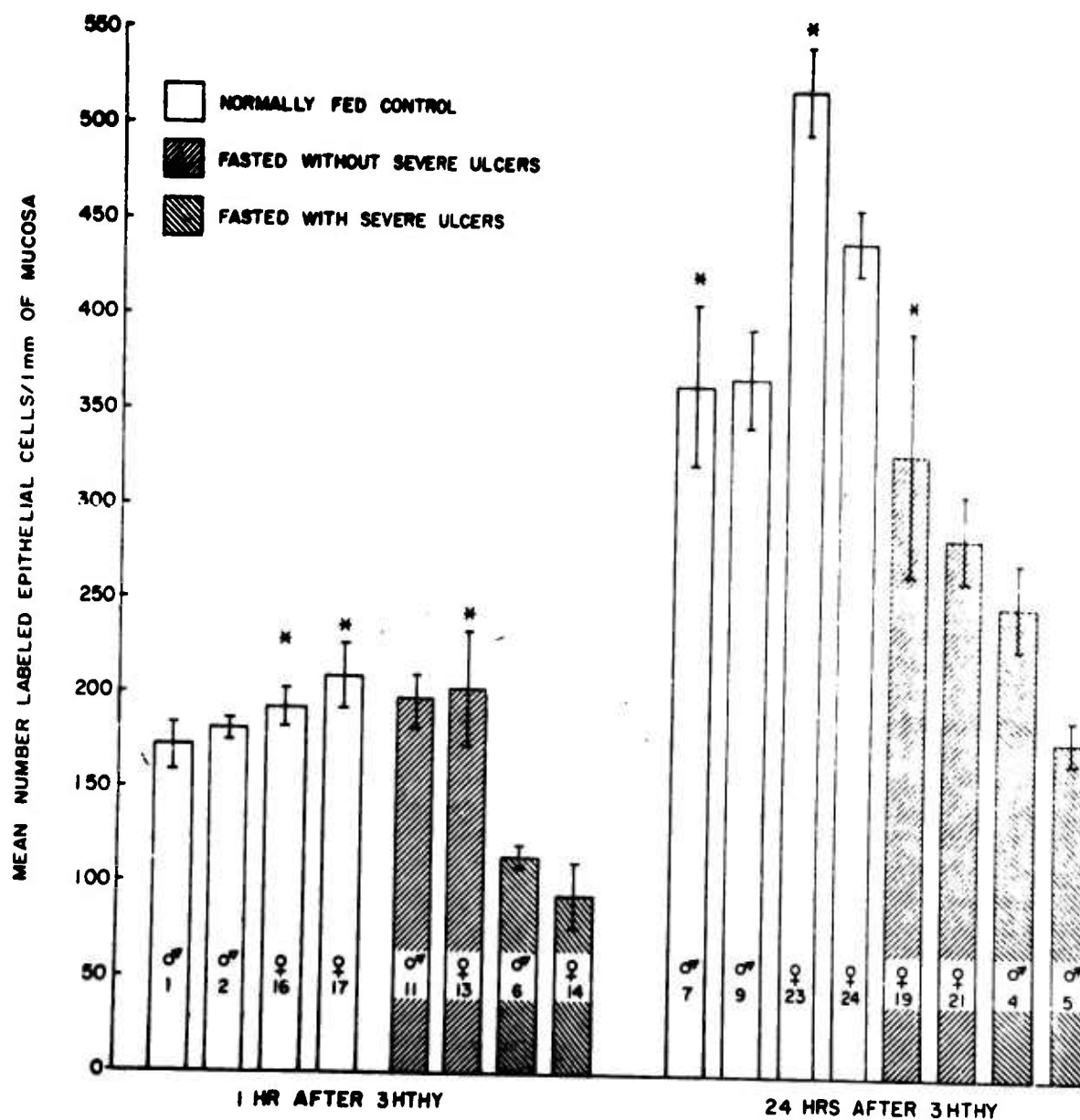
The pathogenesis of acute mucosal ulcers following 1-6 days of fasting was investigated in Walter Reed or Hartley's guinea pigs. Animals were deprived of food but not of water and were sacrificed at daily intervals. The gross appearance of the mucosa was examined after processing for the demonstration of alkaline phosphatase activity (Jervis 1965). Histologic and histochemical study of mucins (Sheahan et al 1970) were carried out on 10% buffered formalin-fixed and routinely processed paraffin

TABLE II
ENZYME HISTOCHEMISTRY OF THE GUINEA PIG DUODENUM

<u>Enzymes</u>	<u>Location</u>	<u>Normal Activity</u>	<u>Changes due to Fasting</u>
Alkaline phosphatase	Striated border Brunner's glands	+++ -	Slight decrease -
Leucine aminopeptidase	Striated border	+	Decrease
Acid phosphatase	Villi epithelial cells Apical cytoplasm Brunner's glands Macrophages	++ - +++	Slight decrease - Increase
Glucose-6-Phosphatase	Villi epithelial cells Cytoplasm Brunner's glands	+++ -	Decrease -
Succinic D	Villi epithelial cells Cytoplasm Brunner's glands	++ -	Decrease -
DPNH diaphorase	Villi epithelial cells Cytoplasm Brunner's glands	+++ +++	Slight decrease Slight decrease
TPNH diaphorase	Villi epithelial cells Cytoplasm Brunner's glands	+++ +	Slight decrease Possibly a slight increase
MAO	Villi epithelial cells Cytoplasm Brunner's glands	+++ traces	Decrease -

The activity of the α -glycerophosphate, β -hydroxybutyric and glutamic dehydrogenases in control animals was so slight that it was not possible to determine changes due to fasting.

3HTHY UPTAKE IN UPPER DUODENUM OF CONTROL GUINEA PIGS AND OF GUINEA PIGS FASTED 5 DAYS



sections. Histochemical study of mucosal enzymes and fat was carried out on fresh frozen sections processed according to techniques well-established in this laboratory (Kent et al 1966) for the demonstration of the following enzymes: alkaline and acid phosphatase, glucose-6-phosphatase, reduced DPNH and TPNH diaphorases, succinic dehydrogenase, glucose-6-phosphate dehydrogenase, alpha glycerophosphate dehydrogenase, beta-hydroxybutyric dehydrogenase, glutamic dehydrogenase, leucine aminopeptidase, monoamine oxidase.

Cell renewal in the mucosa was studied by means of autoradiographic preparations of mucosal strips from control and starved guinea pigs given tritiated thymidine ($^3\text{H}\text{THY}$) intraperitoneally ($1\mu\text{Ci}/\text{gr}$ body weight) either 1 or 24 hours before sacrifice. The pH of luminal content was measured by introducing a unipolar electrode into the stomach and the upper duodenum.

Fasted animals lose weight very rapidly. Shortly after the beginning of fast, the duodenal mucosa develops longitudinal lesions characterized by severe mucosal atrophy accompanied by almost complete mucus depletion in the Brunner's glands, reduction of mucosal enzymatic activity (Table 2), and followed by ulcerations. After 5 days of starvation, there is at least 80% incidence of ulcerations which may perforate by the 6th day. Starvation does not significantly alter the pH content of the stomach or the duodenum, although there is a tendency to higher acidity in the luminal content of many experimental animals. Autoradiographic studies of the bowel (Fig. 17) show that: a) starved animals have similar $^3\text{H}\text{THY}$ uptake in both ulcerated and non-ulcerated areas, the latter showing a normal looking but much thinner mucosa, b) starved animals which showed mucosal atrophy with severe ulcerations have a significantly reduced $^3\text{H}\text{THY}$ uptake throughout the gut, when compared to control or fasted but non-ulcerated animals. The latter, in the jejunum and ileum, have a much thinner mucosa but no villus atrophy.

These observations indicate that the initial duodenal atrophy is not due to a focal slowdown in cell renewal but rather to other local factors such as mucosal irritation by gastric juices which cannot be protected by the action of the depleted Brunner's glands. In the later period, decreased epithelial cell renewal, presumably due to protracted fasting, and also accelerated epithelial cell desquamation, possibly stimulated by persistent gastric juice irritation, result in altered mucosal integrity which leads to mucosal ulceration in the duodenum.

B. Studies on Pathogenetic Factors of Experimental Stress Ulcer

The objective of this study was to clarify pathogenetic factors of experimental stress ulcer and to formulate effective preventive and therapeutic measures.

Gastric microvasculature in the normal rat has been studied by the use of a 2-color silicone rubber perfusion technique (Reynolds et al 1967). The glandular portion of the rat stomach generally shows the same vascular arrangement as that of the human stomach. As seen in Figure 18, arteries, upon penetrating the serosa and the muscularis, form a plexus in the submucosa. Those arteries which run just beneath and parallel to the muscularis mucosae are called distributing arteries. They give off numerous arterioles (connecting arterioles), which penetrate the muscularis mucosae and immediately branch into capillaries at the base of the gastric glands. The mucosal capillaries enclose individual gastric glands by their meshwork, run upward, and, near the mucosal surface, connect with collecting venules which are distributed regularly among the gastric glands. Collecting venules descend the depth of the mucosa in a straight line, penetrate the muscularis mucosae, and connect with collecting veins, the venous counterpart of distributing arteries in the submucosa. Existence of arteriovenous anastomatic channels measuring from 20-40 μ in size are demonstrated for the first time between the arteries and veins of the submucosal plexuses of the rat stomach.

To study gastric microvascular changes, rats were subjected to rotational stress at 60rpm for varying periods (Hase & Scarborough, 1971). Their gastric microvasculature was studied either by in vivo India ink injection or postmortem silicone rubber perfusion. Gastric mucosa from 30 minute-stressed rats shows generalized ischemia. Gastric mucosa from 2-hour rats, on the other hand, show focalization of ischemic and well-circulated areas. In ischemic foci, the entire thickness of the mucosa above the level of connecting arterioles shows filling defects by the silicone rubber perfusion, suggesting the connecting arterioles as the blockage site of mucosal circulation. Signs of microvascular breakage begin appearing in ischemic mucosal foci at this stage.

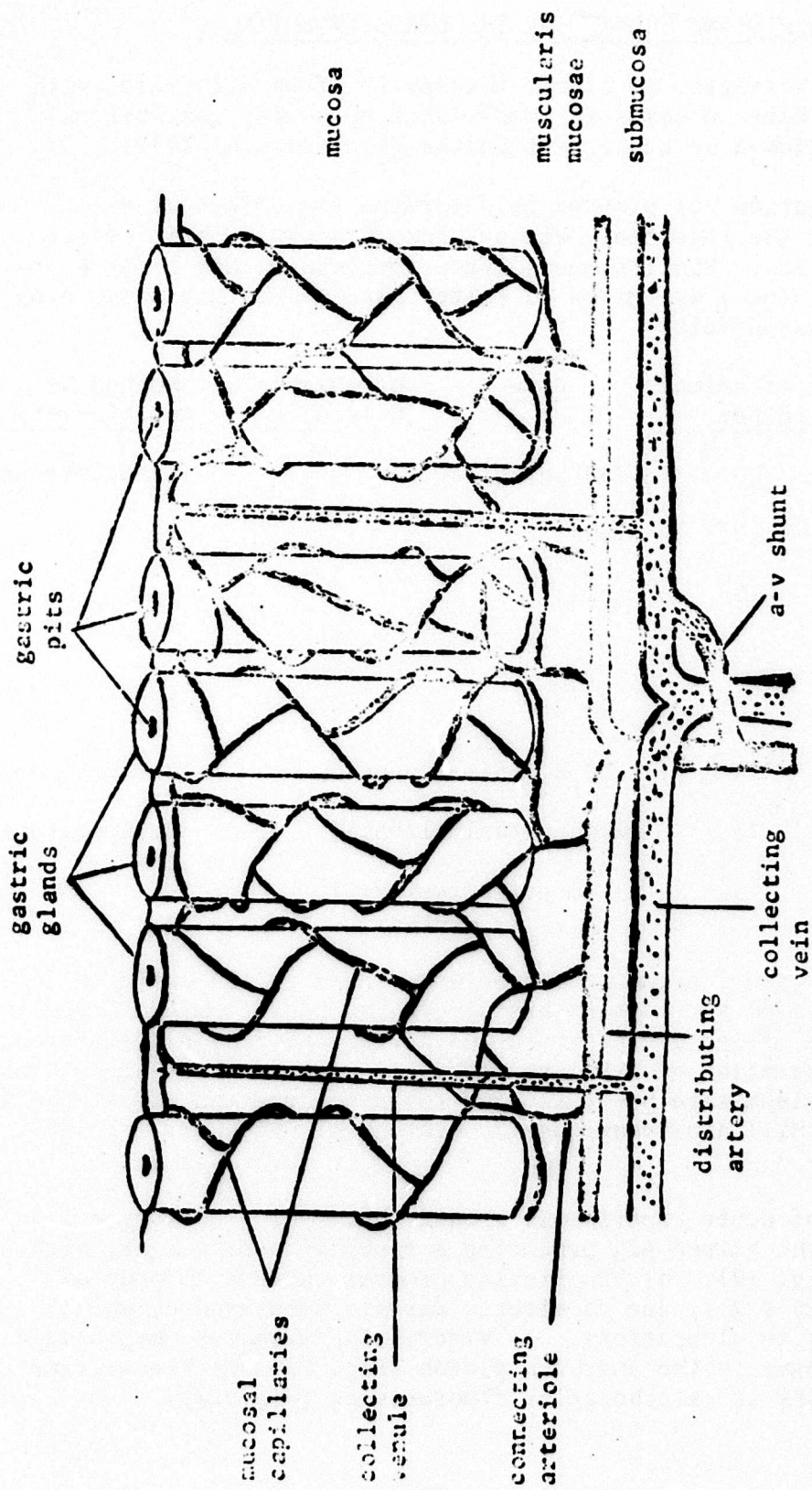
Once lesions appear, the mucosal microcirculatory pattern seems to become increasingly irregular. In stomachs of 4 and 6 hour-stressed rats, small lesions lie in relatively ischemic areas and are accompanied by little hemorrhage; whereas, large lesions are surrounded by an engorged mucosa and are associated with severe hemorrhage. It is possible that, with the initiation of mucosal lesions, various vasoactive substances are liberated (Guth & Kozbir 1968), which, in turn, affect the local circulatory pattern.

In conclusion, mucosal microvascular changes in the development of stress ulcer are as follows:

1. Gastric mucosa of rats in the acute stress initially show generalized ischemia, which become increasingly focal and irregular in later periods.

2. Mucosal ischemia in stress seems to occur as the result of microcirculatory blockage at the level of connecting arterioles. It is most likely caused by contraction of connecting arterioles under influence of the sympathetic nerve.

3. Focalization of mucosal ischemia with consequent intermingling of ischemic and well-circulated areas appears to be important for the development of stress ulcer. In the presence of prolonged ischemia, surface regions of the gastric mucosa are particularly vulnerable to hypoxic change, because the surface mucosa lies at the venous end of the mucosal capillary flow. Protective capacities of mucus and surface epithelium may also be altered in the ischemic areas so as to allow the areas vulnerable to erosive action of gastric juice secreted from adjacent mucosa. Focalization of different circulated areas in the gastric mucosae in the later periods of stress is thought to occur as the result of increasingly disharmonious control of the gastric functions by the autonomic nervous system.



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IV. EFFECT OF VINCRISTINE SULFATE ON THE SMALL INTESTINE

The use of vincristine in cancer therapy is often associated with neuropathy and in certain cases is complicated by severe gastrointestinal symptoms followed by acute peritonitis (Kingry et al 1973).

This investigation was planned to determine the effect of vincristine solely on the intestine, without possible synergistic effect of other therapeutics. Vincristine sulfate (Oncovin R, Eli Lilly & Co., Indianapolis, Ind.) was given to Walter Reed guinea pigs according to the schedule listed below:

<u>Experiment #</u>	<u># of Animals (Exper)</u>	<u>Dose</u>	<u># of Animals (Control)</u>	<u>Method of Administration</u>
I Group 1	10	250 µg/kg/day	2	Intraperitoneally
Group 2	10	100 "	2	"
Group 3	10	50 "	2	"
Group 4	10	25 "	2	"
Group 5	10	10 "	2	"
II	14	200 µg/animal/once	5	Intravenously
III	16	2000 µg/animal/once	4	Intraperitoneally
IV	5	50 µg/kg/weekly/ 12 weeks	1	"

Samples from ileum, cecum and colon were fixed in 10% buffered formalin and processed for routine histology. Samples from the muscular coats of the ileum, fixed overnight in 10% buffered formalin, were processed for the demonstration of alkaline phosphatase in the Auerbach plexus. Samples from the ileum were processed for the demonstration of catecholamines by the Falk Hilliarp technique (J. Histochem. Cytochem. 10: 348, 1962).

The results of acute experiments showed that: a) Vincristine is highly toxic for the guinea pig producing a rapid loss of weight, high mortality rate (Fig. 19). b) Vincristine produces severe atrophy of the mucosa (Fig. 20 & 21), due to mitotic arrest in metaphase, which subsequently leads to ulcerations. c) Vincristine produces morphologic and enzymatic changes in the Auerbach plexus (Fig. 22). d) Vincristine produces marked loss of catecholamine fluorescence (Fig. 23).

In the chronic experiment, mucosal and neural changes were present, but to a much slighter degree than in the acute experiments.

In conclusion, vincristine appears to have a two-fold effect on the bowel; it may produce ulcerations due to progressive mucosal atrophy resulting from arresting mitosis of mucosal cells and it may interfere with gut motility and blood flow through neuronal impairment. Changes observed may explain gastrointestinal complications, such as perforations and adynamic ileus, observed occasionally in cancer patients during vincristine therapy.

Mortality In Vincristine Treated Guinea Pigs

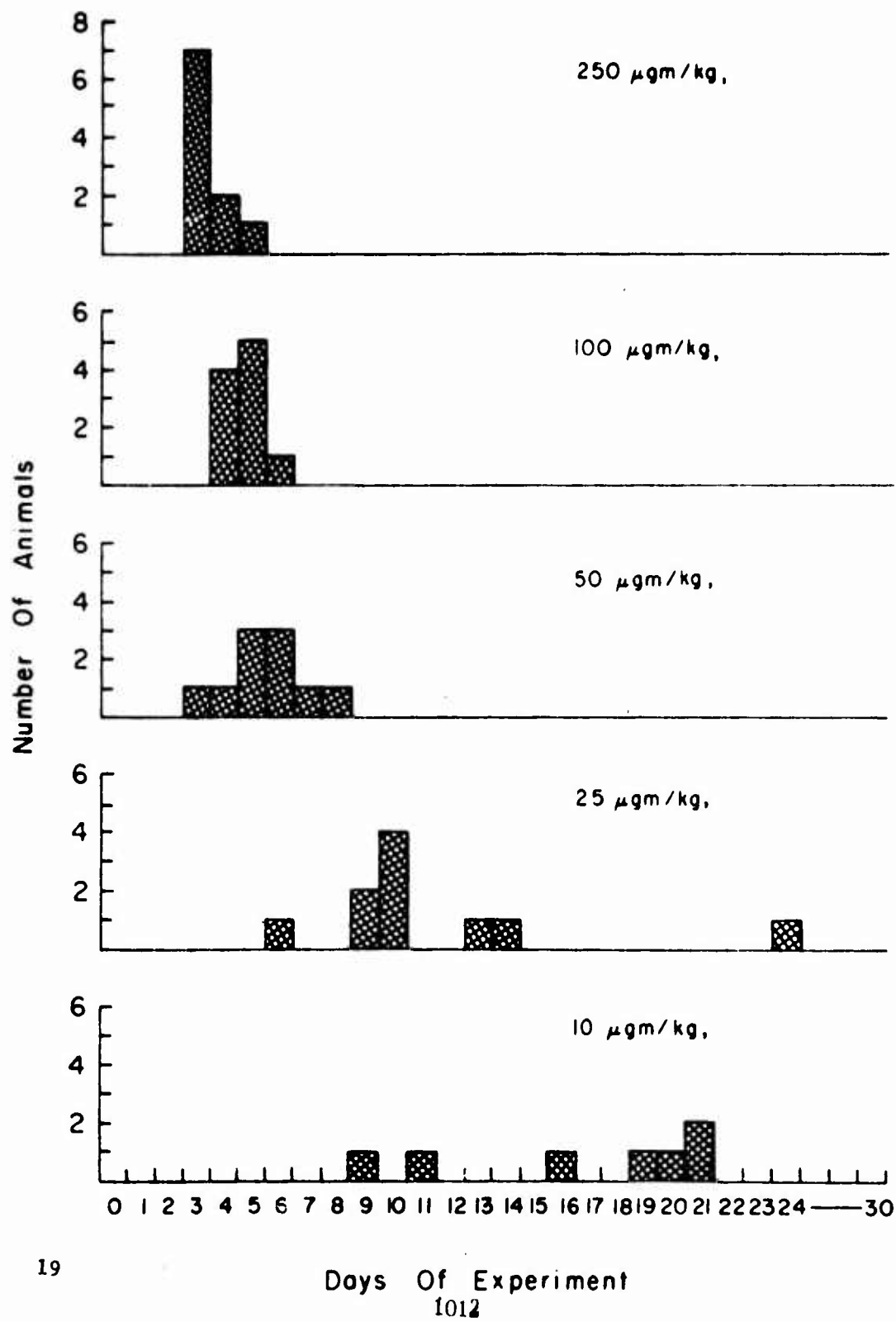




Fig. 20 - Ileal mucosa: Experimental Day 3.



Fig. 21 - Ileal mucosa: Control.



Fig. 22 - Alkaline phosphatase activity in Auerbach plexus.



Fig. 23 - Catecholamines, fluorescence.

V. STUDIES ON KIDNEY PATHOLOGY

Obstructive nephropathy is a frequent complication arising from partial or complete occlusion of the urinary tract. Occlusion may occur in any portion of the urinary tract and may result from different causes. The occlusion may result in progressive destruction of the kidney. The pathogenesis of the renal changes are currently incompletely understood and adequate clinical and morphological criteria with which to recognize the extent of renal damage are not available.

The present study was initiated to relate the physiological changes of acute ureteral obstruction in the rabbit with morphological alterations seen by light and electron microscopy as well as the changes in cellular enzyme activity studied by histochemistry. Particular emphasis has been placed on the changes which occur within the renal interstitium because they are related to the subsequent fibrosis and tubular atrophy which leads to irreversible loss of renal function.

Complete unilateral ureteral obstruction was produced in male New Zealand white rabbits by tying a suture around the right ureter just above the ureterovesicle junction.

The function of each kidney was separately examined on days 1, 4, 7, 16 and 32 after ligation of one ureter as well as in two normal animals. Water was removed 20 hours before the tests of renal function were conducted.

Each ureter was catheterized and separate urine collections were begun. A constant perfusion solution of 50% glucose and 0.5 μ Ci of 125 I Iothalamate and Pitressin was administered intravenously. Urine samples were collected from each ureter at 10 minute intervals and their volumes were accurately determined. Blood samples were obtained by cardiac puncture. The radioactivity of blood and urine Iothalamate was determined by gamma counting. The osmolarity of urine and serum were determined on an Advanced Osmometer. Urine and serum glucose levels were determined with an autoanalyzer by the ferrocyanide method. From this data, the Iothalamate clearance (GFR), osmolar clearance (Cosm), net free water reabsorption (TCH₂O) and glucose reabsorption (T_mglucose) were calculated.

Morphological studies were performed on all animals which included control and animals obstructed 1 and 4 days. Fixation, except in those animals used for histochemical study, was by in vivo perfusion of one-half strength Karnovsky's solution. Thin, 1 mm, sagittal sections were further fixed and processed for EM.

Histochemical Studies: For histochemistry, control and animals obstructed 1 and 4 days were killed. Histochemical reactions were performed on frozen sections to demonstrate the activity of a number of enzymes and the presence of fat (Table 3). Each level of the nephron was graded semiquantitatively from 0 (no reaction) to 4+ (prominent reaction) by two separate investigators

Results: The glomerular filtration rate (GFR) was reduced in the obstructed kidney to approximately 50% of the contralateral kidney GFR within 24 hours of the obstruction ($P < 0.005$). By the fourth day the GFR was less than 2 mg/min or about 30% of the contralateral GFR ($P < 0.001$).

Proximal tubule function measured as capacity for tubular reabsorption of glucose fell from a mean of 48mg/min to 30mg/min after 24 hours and to less than 10mg/min after 4 days of obstruction. When these values are corrected for GFR, there was no fall in T_m glucose until the 7th day and 15th day after obstruction. The obstructed kidney lost the ability to concentrate urine within the first 24 hours ($P < 0.001$). By light microscopy, the glomeruli were observed to be congested on day 1, but otherwise normal.

In the proximal convoluted tubules at 1 day, EM revealed collapsed lumina and an intact brush border except focally where swollen apical blebs of cytoplasm protruded into the lumen. The apical cytoplasm showed a decrease in tubular invaginations and vesicles and numerous large membrane-limited bodies, as well as scattered electron dense tubules. Mitochondria were rounded and many had lost their vertical orientation to the basement membrane (Fig. 24 & 25). On the fourth day these changes were more prominent and there was thinning and focal loss of the brush border.

Descending thick segments at day 1 had patent lumina, intact brush borders, although there was extensive vacuolation of the cytoplasm. By the 4th day, the cells revealed numerous membrane-limited bodies, thinning of the surface microvilli, and loss of basal interdigitation (Fig. 26). The thin limb revealed no significant changes at day 1 or 4 except near the tip of the papilla where, at day 4, there was obvious necrosis. Occasionally, their epithelium contained numerous lipid droplets (Fig. 27).

Ascending thick segment revealed, at day 1, slight swelling of mitochondria, markedly decreased basilar interdigitation, basal lipid droplets, and increased rough ER. The lumen contained an occasional protein cast. These changes were more prominent at four days (Fig. 28).

Distal convoluted tubules at days 1 and 4 were dilated and, in some, the epithelium appeared flattened.

Collecting ducts were dilated throughout their course after 1 day of obstruction and, in the medullary rays and cortex, they were lined by a flattened epithelium (Fig. 24 & 29), with widening of the intercellular space which was often invaded by mononuclear cells. The junctional complexes at the cell surfaces were intact.

At the tip of the papilla, the transitional epithelium was intact but there was necrosis of the underlying cells lining the collecting ducts (Fig. 30).

Cortical collecting ducts at 4 days remained widely dilated and contained an occasional cell (Fig. 31). At the papillary tip, there was more extensive necrosis of the tubular epithelium.

The interstitial space was enlarged after 1 day of obstruction, particularly near the collecting ducts (Fig. 24). The interstitial spaces contained three main cell types: altered interstitial fibroblasts, occasional mononuclear cells, and extravasated red blood cells. In addition, polymorphonuclear leukocytes were seen margined within the capillaries. The interstitial fibroblasts were very enlarged. Their processes often were wrapped around smaller mononuclear cells.

At the 4th day of obstruction, the interstitial space was further enlarged (Fig. 32). The cortical fibroblasts were further enlarged with increased rough-surfaced endoplasmic reticulum, dilated cisternae containing electron dense material and prominent Golgi apparatus (Fig. 32 & 33). The interstitium contained many more mononuclear cells than previously. The ground substance of the cortex contained an increased amount of finely flocculent electron dense material (Fig. 34).

There was variable congestion of the glomerular and peritubular capillaries. The capillaries within the outer medulla were consistently congested and occasional small microthrombi containing fused platelet aggregates were seen within the inner medulla (Fig. 35).

At the 4th day also, the larger veins were dilated. Not infrequently, polymorphonuclear leukocytes were margined along the capillary endothelium.

The distribution of enzyme activity in normal rabbit kidneys is seen on Table III. Table IV shows the major changes in these activities occurring with obstruction of the ureter.

Discussion

Few previous studies on the effects of ureteral obstruction have attempted to correlate physiological and morphological data. Much of what is known physiologically concerning the effects of obstruction in the kidney deals with the very acute changes, while most pathologists are familiar only with the effects of chronic obstruction.

These studies have revealed no anatomic lesion in the vessels or glomeruli which could explain the reduction in glomerular filtration observed. Although the majority of proximal tubules appeared after in vivo perfusion to be abnormally collapsed, this may reflect decreased filtered load. A second factor in the reduction of glomerular filtration could be decreased net filtration pressure.

Ultrastructural and histochemical changes in the proximal tubule were seen to coincide with functional changes related to T_m glucose.

The loss of concentrating ability following acute ureteral obstruction as seen in this study has also been observed in man, dog and rat. The present study raises a number of questions. First, there seems to be decreased GFR and, therefore, in the nephrons, a decreased osmotic load is delivered to the distal nephron which would expect to interfere with the maintenance of the tissue osmotic level. Secondly, the ascending thick segment of the nephron, where sodium ion is actively pumped from the nephron into the interstitium, is deranged suggesting a decreased transport of sodium ion into the interstitium. The finding of coagulation necrosis at the papillary tip, where the maximal tubular tissue osmotic gradient usually exists, and occasional microthrombi within the vasa recta support the idea that medullary perfusion is deranged. Further studies are needed to ascertain to what extent each of these lesions contributes to loss of renal concentration.

Changes in cellular metabolism might be expected in view of the functional and morphological changes observed in the early phases of acute ureteral obstruction and of the striking changes in the activity of various enzymes within the cells, which correlated with morphologic changes. Increased activity of acid phosphatase in the proximal tubule reflected increased prominence of the membrane-bound lysosomes and the decreased reaction of alkaline phosphatase in the proximal tubules and pars recta reflected the loss of the brush border and decreased glucose reabsorption. A small population of proximal tubules retaining strong alkaline phosphatase reaction even on the 4th day of obstruction could be correlated with the occasional normal tubule that was seen by electron microscopy.

It is clear from these studies that acute ureteral obstruction leads to a complex series of events which affects virtually all segments of the nephron. This is reflected in biochemical and morphologic alterations of the component parts of the nephron which ultimately are reflected in deranged function. The extent to which these early changes are reversible is not known and will require further study. The implication of the work, however, is that complete ureteral obstruction leads to considerable damage to the mammalian kidney even in the early stages.

TABLE III
NORMAL DISTRIBUTION OF ENZYME ACTIVITY IN RABBIT NEPHRON

	Proximal Convoluted Tubule	Descending Thick Segment	Thin Limb	Ascending Thick Segment	Distal Convoluted Tubule	Collecting Duct	Glomerulus
Acid Phosphatase	2-3	3-4	1	1-2	1-2	2	0
Alkaline Phosphatase	4	4	0	0	0	0	0
ATPase	2-4	2-3	0	2	2-3	0	0
Glucose-6-Phosphatase	4	3	0	0	0	0	0
Succinic Dehydrogenase	3-4	3-4	0	3	1-4	0	0
Monamine Oxidase	3	3	0	1-3	1-2	0	1
Leucine Amino- peptidase	2-4	2-4	0	0-1	0	0	0
DPNH Dehydrogenase	4	4	2	4	4	3	1
TPNH Dehydrogenase	4	4	1-2	3-4	4	3	1
Alpha-Glycero Phosphate Dehy.	0	3-4	1	3	0	3-4	1
Glutamic Dehy.	2	2	0-2	1-2	2	1-3	0
Glucose-6-P.-Dehy.	1	2-3	0	0-2	2-3	1	0
Beta-Hydroxy Butyric Dehy.	1	3	0-1	4	4	3	0
Oil Red O	0	0	0	0	0	0	0

Enzyme Activity Graded from 0 (no activity) to 4+ (intense reaction).

TABLE IV
CHANGES IN ENZYME ACTIVITY WITH OBSTRUCTIVE NEPHROPATHY

	Proximal Convoluted Tubule		Descending Thick Segment		Thin Limb		Ascending Thick Segment		Distal Convoluted Tubule		Collecting Duct		Glomerulus	
	1	4	1	4	1	4	1	4	1	4	1	4	1	4
Acid Phosphatase	+	+	-	-	-	-	-	-	-	-	-	-	-	-
Alkaline Phosphatase	+	++	+	++	-	-	-	-	-	-	-	-	-	-
ATPase	+	++	+	++	-	-	-	+	-	-	-	-	-	-
Glucose-6-Phosphatase	-	+	+	+	-	-	-	-	-	-	-	-	-	-
Succinic Dehydrogenase	+	+	+	++	-	-	+	+	+	+	-	-	-	-
Monamine Oxidase	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Leucine Amino-peptidase	+	+	+	+	-	-	-	-	-	-	-	-	-	-
DPNH Dehydrogenase	-	+	-	+	-	-	-	+	-	-	-	-	-	-
TPNH Dehydrogenase	-	+	+	+	-	-	-	+	-	-	-	+	-	-
Alpha-Glycero Phosphate Dehy.	-	-	+	++	-	-	-	+	-	-	+	+	-	-
Glutamic Dehy.	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Glucose-6-P.-Dehy.	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Beta-Hydroxy Butyric Dehy.	-	-	+	++	-	-	-	-	-	-	+	+	-	-
Oil Red O	-	-	-	-	-	-	-	-	-	-	-	+	-	-

Arrows indicate areas in nephron with significant change in enzyme activity.
+ = 1+ decrease, ++ = 2+ decrease, a dash indicates no significant change.
Each segment of the nephron is graded for each enzyme on days 1 and 4.



Fig. 24-Renal cortex after 1 day. Note collapse of some proximal tubules, lined with finely vacuolated cells. A cast is seen in distal tubule. Note increase in interstitium around collecting ducts which have dilated lumina. Toluidine blue, X220.
 Fig. 25-Proximal convoluted tubule with ocluded lumen. Note rounded mitochondria (arrows) enlarged lysosomes (L), decreased apical tubular invaginations and vesicles, and loss of basal interdigitation and of mitochondrial orientation. X6200.



Fig. 35-Capillary from inner medulla after 4 days. Note partially fused platelet aggregates (PA) between the red cells and adherent to the endothelial cells. X8100.

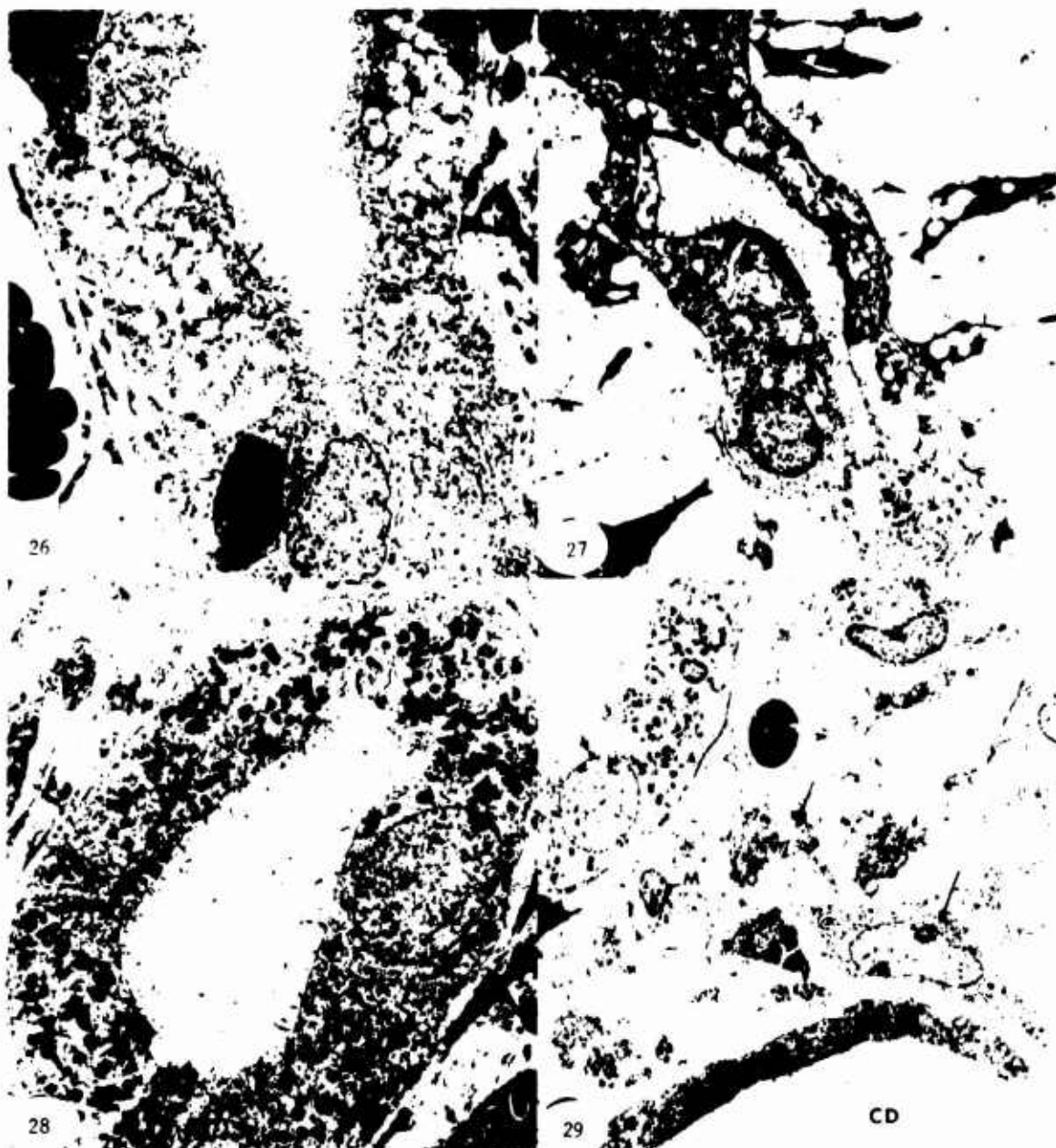


Fig. 26-Descending thick segment after 4 days. Note thin microvilli, dilated lysosomes and loss of basal interdigitation. X2900.
 Fig. 27-Thin limb after 4 days. Note lipid droplets (arrows). X3400.
 Fig. 28-Thick ascending segment after 4 days. Mitochondria appear rounded. X3300.
 Fig. 29-Cortex after 4 days. Note dilated collecting duct (C.D.). Mononuclear cell (M) and interstitial cells (arrows) are present in enlarged interstitium. X2100.



30

Fig. 30-Papilla after 1 day. Note focal necrosis of the epithelium lining collecting ducts which may be lifted off the basement membrane (arrows), D indicates debris. H&E, X150.



Fig. 31-Collecting duct after 4 days. Intercellular spaces (IS) contain mononuclear cells. X10,000.

Fig. 32-Cortex after 4 days. Top cell is an interstitial fibroblast. Note dilated cisternae in ER and prominent Golgi region. Arrow indicates cell junction. Bottom cell is a monocyte characterized by clumped heterochromatin and cytoplasm with ribosomal rosettes but few organelles. X20,200.



Fig. 33-Cortical interstitium after 4 days. Stimulated interstitial cells (IC) showing relationship to mononuclear cells (M). Note the nuclear chromatin and the prominent rough-surfaced ER in fibroblasts. X4600.
 Fig. 34-Inner medulla after 4 days. Note (arrows) slightly flocculent material which is displacing the normal interstitial ground substance. X6200.

B. Smooth Muscle Characteristics in Renal Interstitial Cells

The classic view that, with fibrosis, contraction resulted from the shortening of collagen was seriously questioned when it was shown that wounds in scorbutic animals could contract independently of collagen formation. Recently, it has been demonstrated that fibroblasts within healing wounds developed cytoplasmic filaments and dense plaques similar to those characteristic of smooth muscle and also developed antigens which cross-reacted with anti-smooth muscle antibody by immunofluorescence study.

In our previous studies, the renal interstitial cells have been shown at EM levels to resemble inactive fibroblasts connected through a series of junctions. The present study was undertaken to follow, by fluorescent antibody and electron microscopy, the changes which occur in these cells following complete unilateral ureteral obstruction.

Our model of complete unilateral ureteral obstruction in rabbits was used. Specimens from kidneys of control rabbits and of rabbits obstructed 24 hours and 4, 7, 16, and 32 days were quenched at -160°C for antibody immunofluorescence study or they were processed for electron microscopy by standard technique.

Cryostat sections of fresh frozen kidneys were covered with human sera containing anti-smooth muscle antibody (HASA) and reacted for 45 minutes at room temperature. The sections were then washed 3 times with phosphate buffered saline (PBS), pH 7.2, and then reacted with fluorescein-labelled goat anti-human gamma globulin. The fluorescein labelling of the interstitial cells of both the cortex and medulla were graded from 0 (no reaction) to 4+ (cytoplasm with diffuse positive fluorescence).

The antisera used for immunofluorescent characterization was obtained from patients with chronic active hepatitis. These antisera have been shown to have specific activity for smooth, but not skeletal or cardiac muscle in humans, rabbits and rats. Smooth muscle areas tested were: rat stomach, human myometrium and rat, rabbit, and human vascular smooth muscle. Ultrastructural localization of the tissue antigen by use of the peroxidase labelling technique indicated that antibody binds to the filamentous bundles within the smooth muscle cell cytoplasm.

The results of the reaction of the fluorescein-labelled anti-smooth muscle antibody with the interstitial cells are summarized in Table V. Following 8 days of obstruction, there was a marked increase of the labelling of the cortical and medullary interstitial cells (1-3+). By the 16th day and the 32nd day, the long cytoplasmic processes of the interstitial cells were intensely labelled (3-4+) (Fig. 36-37).

At the EM level, the control interstitial cells appeared as inactive fibroblasts having a Golgi apparatus in the perinuclear region, a scant amount of rough-surfaced endoplasmic reticulum, and long cytoplasmic processes (Fig. 38). The cytoplasmic processes occasionally contained filamentous bundles just beneath the plasma membrane and the processes of neighboring cells were closely related and separated by a constant gap.

By the 7th day of obstruction, the interstitial space was enlarged and contained numerous interstitial fibroblasts. The fibroblasts appearing in the cortex and outer medulla had a central, oblong nucleus and numerous long stellate cytoplasmic processes. Ultrastructurally, the cells had prominent nucleoli, well-developed Golgi apparatus, and greatly increased rough-surfaced endoplasmic reticulum. The most interesting change was the presence of numerous filaments and dense plaques seen at the periphery of the cells and particularly in the cytoplasmic processes.

By the 16th day of obstruction, the fibroblasts were more numerous in both the cortex and medulla, the evidence of diffuse fuchsinophilic fibers was clearly present. The cells in the cortex revealed elongated processes which frequently made close contact with similar processes from neighboring cells (Fig. 39). Processes were separated by 250-300 Å gaps. The peripheral cytoplasm of these fibroblasts had a striking increase in the number of filaments measuring approximately 55-70 Å in diameter. These filaments were arranged in a parallel fashion and were inserted into dense plaques similar in appearance to those characteristic of smooth muscle (Fig. 40). The perinuclear areas revealed a prominent Golgi apparatus. The rough-surfaced endoplasmic reticulum was greatly increased. The medullary fibroblasts revealed similar, though less dramatic changes.

By the 32nd day of obstruction, the interstitial fibroblasts were separated by increased amounts of collagen. Long cytoplasmic processes could be seen coursing through the group of collagen fibers and surrounding the duplicated basement lamina of the atrophic tubules. The peripheral filaments were present, but were less prominent than those seen at 16 days. There were numerous, coated vesicles in the cytoplasm and several fibroblasts were seen to contain myelin figures.

The present work indicates that the interstitial cells of the kidney are similar to fibroblasts seen in granulation tissue in that they have the capacity to synthesize numerous cytoplasmic filaments and to develop an antigen or antigens which cross-react with antibody specific for smooth muscle.

The filaments and the antigen are most likely related. The filaments measure 60-75°A and are similar in morphologic appearance to actin seen in smooth muscle and correspond to the filaments described in activated fibroblasts of healing wounds. Three types of filaments occurring in 3T3 fibroblasts grown in confluent cultures have been described: (1) Alpha filaments measuring 60-80°A in diameter arranged in parallel bundles within the ectoplasm and associated with dense regions (2) Beta filaments which are 100°A in diameter and tend to form loose fascicles within both the ectoplasm and endoplasm (3) Microtubules, 250A in diameter present throughout the cytoplasm. The filaments seen in the renal interstitial cells appear to correspond to the 60-80°A alpha filaments of McNutt.

Several important factors have been ascribed to these filaments such as control of cytoplasmic viscosity; participation in contractile mechanism and participation in cell-to-cell contacts. These filaments have been observed in certain non-muscle cells to bind heavy meromyosin to form arrowhead complexes suggesting a relationship to the F-actin of muscle cells.

The smooth muscle antibodies which develop in human patients suffering chronic active hepatitis have been shown to react with a variety of vascular and visceral smooth muscle. The exact nature of the antibodies is not known; when labelled with peroxidase, they have been shown to bind to the filaments of smooth muscle and thrombosthenin derived from platelets. It is becoming clear that these filaments are an actin-like protein and with proper fixation have been seen in a variety of non-muscular cells. It is of interest that a low level of antibody binding to normal renal interstitial cells occurs and careful examination indicates the presence of the filaments in the peripheral ectoplasm of the normal resting interstitial cells.

Contacts between stimulated renal interstitial cells are similar to those described in fibroblasts of granulation tissue. This is a requirement for an effective contractile system. Preliminary work from our department has indicated that these cells respond by contraction when stimulated in vitro with serotonin, angiotensin II or norepinephrine. This is similar to the response recorded in granulation tissue, but may have special significance in a parenchymous organ such as the kidney.

Conclusion

It is quite conceivable that stimulated renal interstitial cells once present could respond by contraction to local concentrations of catecholamines or other vasoactive substances. This could lead to local changes in the microcirculation, producing ischemia, and could further potentiate the injury. Such a pathogenetic mechanism may in part explain the slowly progressive tubulo-interstitial damage observed in kidney diseases from different causes. We have observed similar cells containing numerous filaments and dense plaques in association with degenerating glomeruli in a variety of chronic human renal diseases. Therefore, further studies on stimulated renal interstitial cells by other parameters will provide additional information on injuries and diseases of the kidney.

TABLE V

<u>No. of Days Obstruction</u>	<u>Number of Animals</u>	Reactivity of Fibroblast with Antismooth Muscle Antibody	
		<u>Cortex</u>	<u>Medulla</u>
0	7	.93 (0-1)	.93 (0-1)
8	5	2.1 (1-3)	2.5 (1-3)
16	4	2.0 (2-2)	3.5 (3-4)
32	5	2.0 (1-3)	3.6 (3-4)

* Fluorescence due to binding of labelled anti-smooth muscle antibody-graded from 0 (no reaction) to 4+ (high reactivity), expressed as mean (range).



Fig. 36-Normal rabbit cortex showing indirect immunohistochemical staining with HEMA. Antibody is bound to smooth muscle of interlobular artery, afferent arterioles and mesangium. Interstitial cells are almost reactive. X100.

Fig. 37-Rabbit cortex after 16 days of ureteral obstruction showing indirect immunohistochemical staining with HEMA. The antibody is bound to the stimulated interstitial cells. X100.

Fig. 38-Normal rabbit cortical interstitial cell. Note the junction between cytoplasmic processes of adjacent cells (arrow). X11,000.

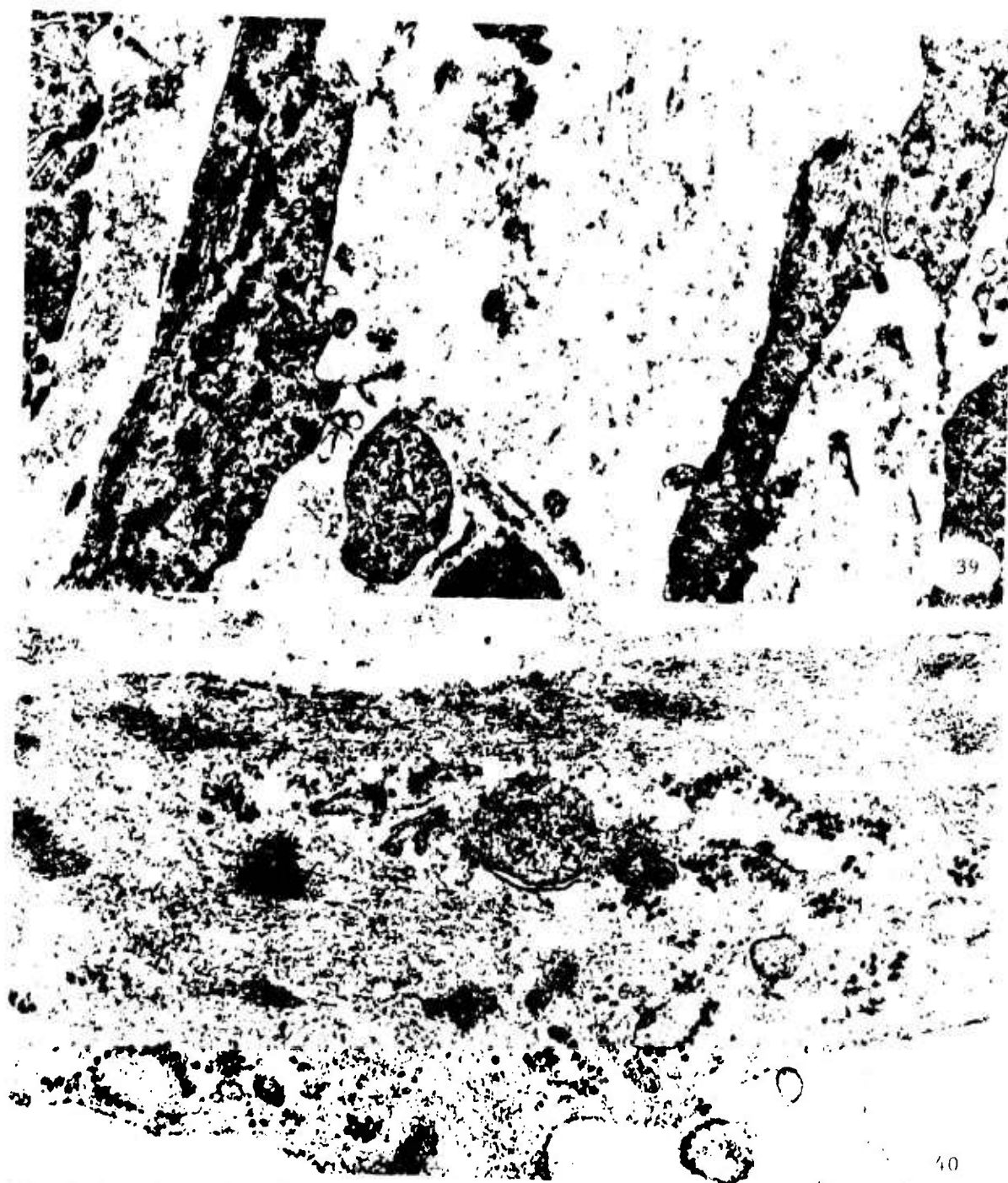


Fig. 39-Rabbit renal cortex after 16 days. Note long processes of stimulated interstitial cells. The cytoplasm contains numerous parallel filaments (at right). Note junction between the processes of two cells. X22,000.
 Fig. 40-Peripheral cytoplasm of stimulated cortical interstitial cells after 16 days. Note numerous filaments inserting in dense plaques. X60,000.

C. Glomerulonephritis in Experimental African Trypanosomiasis

When infected with protozoa, such as malaria and babesia, mammals, including man and monkeys, may develop glomerulonephritis. These studies were spurred by the findings that monkeys injected with a trypanosome strain, originally isolated from a patient, developed chemical and histologic evidence of renal failure and glomerulonephritis. Some of these kidneys contained the morphologic changes similar to those described in human cases of membranoproliferative glomerulonephritis. This study was undertaken to clarify glomerulonephritis induced by the trypanosoma infection in the monkey.

Sixteen rhesus monkeys were each inoculated intravenously with 10,000 organisms of Trypanosoma rhodesiense (EATRO #1886 strain) contained in 0.5ml of phosphate-buffered saline-glucose (PSG) solution. Control animals received 0.5ml of PSG solution only. The EATRO #1886 strain was isolated from a human patient in Uganda in 1971 and has since been maintained in the laboratory either by storage at -70°C or by passage in albino rats. Blood obtained by ear puncture from infected monkeys were examined 5 days each week to monitor paraitemia. Blood for serum collection was taken from the femoral veins. The sera were stored at -70°C until used.

Biopsy wedges of renal cortex were taken under anesthesia at 14, 29, or 30 and 50 days following inoculation of trypanosomes.

Fresh frozen cryostat sections were processed for immunofluorescent antibody study. It has been demonstrated that antibodies to human IgG, IgA, IgM, C4 and properdin (Ward 1969) gives a strong cross-reaction to the heterologous monkey serum protein, as shown by double diffusion in gels. Accordingly, antibodies to the human proteins were used. The only exception were antibodies to monkey C3 prepared in rabbits as previously described. In immunofluorescent studies, the direct technique was used for all proteins except properdin, with incubation of tissue sections with the fluorescein-labelled antibody, followed by washing. In the case of properdin, the indirect immunofluorescent technique was employed, incubating tissues first with rabbit antibody to human properdin followed by washing and incubation of the tissue with fluorescein-tagged sheep to rabbit IgG (Ward & Conran 1969).

Serum CH₅₀ levels were assayed with sensitized sheep red cells according to the technique of Kent & Fife. Complement components C3 and C4 were assayed utilizing the cross-reactions of antibodies to the human components with monkey proteins, using the single radial immunodiffusion technique of Mancini et al as modified by Yount et al.

Results: Rhesus monkeys infected with Trypanosoma rhodesiense develop a proliferative glomerulonephritis associated with deposits consisting of the third component of complement (C3), properdin, and, in some cases, IgM. A few renal biopsies contained IgG and C4; none contained IgA. The pattern of deposits as revealed by immunofluorescence was granular. Sera from animals developing glomerulonephritis were hypocomplementemic; by radial immunodiffusion most animals showed depression of C3 but not C4 levels. These findings suggest that the glomerulonephritis associated with trypanosomal infections in monkeys is related to deposition of immunologically important serum proteins, two of which represent reactants in the alternate complement pathway.

D. Histochemical Studies on Transplant Kidneys

During the past 2 years, nephrectomies and kidney biopsies have been collected from 52 patients admitted to Walter Reed General Hospital for kidney transplant, for the purpose of investigating possible changes in fat content and activity of intracellular enzymes of the kidney parenchymal cells. Biopsies were collected at the time of transplant and at 3 months, 1 year and 2 years post-surgery. They were inserted in a slit made on the surface of a mouse kidney, which provided support and a positive control for the enzymatic reactions, and quenched in isopentane at -160°C . Eight μ sections, cut in the cryostat, were stained for histologic study with H&E and for histochemical investigations with PAS, ORO and with the techniques used for the demonstration of the following enzymes: alkaline and acid phosphatases, leucine aminopeptidase, glucose-6-phosphatase, succinic dehydrogenase, DPNH diaphrase, beta-hydroxybutyric and alpha-glycerophosphate dehydrogenase.

In rejected kidneys, there is a severe loss of enzymatic activity which may become evident shortly after transplantation. The correlation of histochemical findings and clinical data is currently in progress.

Project 3A062110A822 MILITARY INTERNAL MEDICINE

Task 00 Military Internal Medicine

Work Unit 123 Histopathologic Manifestations of Military Disease
and Injuries

Literature Cited.

Publications:

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION ^a	2. DATE OF SUMMARY ^a	REPORT CONTROL SYMBOL DD-DR&E(AR)636	
				DA OA 6485	72 08 01		
3. DATE PREV SUMRY	4. KIND OF SUMMARY	5. SUMMARY SCTY ^a	6. WORK SECURITY ^a	7. REGRADING ^a	8. DES'N INST'N	9. SPECIFIC DATA - CONTRACTOR ACCESS	10. LEVEL OF SUM
72 07 01	H. Term.	U	U	NA	NL	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	A. WORK UNIT
11. NO./CODES ^a		PROJECT NUMBER		TASK AREA NUMBER		WORK UNIT NUMBER	
12. PRIMARY		3A062110A822		00		125	
13. CONTRIBUTING							
14. CANCELLATION X		CDOG 114(f)					
15. TITLE (Precede with Security Classification Code) ^a							
(U) Hematology of Nutritional Deficiencies of Military Importance							
16. SCIENTIFIC AND TECHNOLOGICAL AREAS ^a							
002600 Biology 012900 Physiology							
17. START DATE		18. ESTIMATED COMPLETION DATE		19. FUNDING AGENCY		20. PERFORMANCE METHOD	
63 07		72 08		DA		C. In-House	
21. CONTRACT/GRANT				22. RESOURCES ESTIMATE		23. PROFESSIONAL MAN YRS	
A. DATES/EFFECTIVE: NA				PRECEDING		B. FUNDS (in thousands)	
B. NUMS ^a : NA				FISCAL YEAR		72	
C. TYPE:				CURRENT		73	
D. KIND OF AWARD:				72		2	
E. AMOUNT:				73		45	
F. CUM. AMT.				73		45	
24. RESPONSIBLE DOD ORGANIZATION				25. PERFORMING ORGANIZATION			
NAME: ^a				NAME: ^a			
Walter Reed Army Institute of Research				Walter Reed Army Institute of Research			
ADDRESS: ^a				ADDRESS: ^a			
Washington, D.C. 20012				Division of Medicine			
				Washington, D.C. 20012			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)			
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Buescher, COL E. L.				Conrad, COL M. E.			
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				SOCIAL SECURITY ACCOUNT NUMBER: [REDACTED]			
26. GENERAL USE				ASSOCIATE INVESTIGATORS			
Foreign Intelligence Not Considered				NAME:			
				Toskes, MAJ Phillip P.			
				NAME:			
				DA			
27. REVISIONS (Precede EACH with Security Classification Code)							
(U) Diet; (U) Intestine; (U) Iron; (U) Protein; (U) Hemoglobin; (U) Vitamin B12							
28. TECHNICAL OBJECTIVE, 29. APPROACH, 30. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
<p>23. (U) The nutritional anemias are correctable diseases that are commonplace in geographic areas of military importance. These diseases markedly reduce the capability of affected populations to perform work or sustain a military effort and remain self-supporting.</p> <p>24. (U) Establishment of standards and standard methods for detection and quantification of these diseases. Studies of the nutrient content of various foodstuffs and the availability of these nutrients for absorption from these foodstuffs in normal subjects and in populations where nutritional deficiencies and chronic infections are commonplace.</p> <p>25. (U) 72 07 - 72 08 Collaboration was continued with ICSH and WHO in the development of referee methods and standards for the measurement of iron and iron-binding protein. Exocrine pancreatic secretions were shown to be a factor in the absorption of vitamin B12 in humans, and B12 absorption was found to be a sensitive method of detecting pancreatic insufficiency. The characteristics of the blind loop syndrome were defined in collaboration with the Department of Gastroenterology. This work unit terminated as of 1 Aug 72 because of consolidation into another work unit. Studies to be continued under Project No. 3A061102B71R, Work Unit No. 086. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 72 - 30 Jun 73.</p>							

PII Redacted

^a Available to contractors upon originator's approval.

DD FORM 1498

PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A 1 NOV 66 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION ^a	2. DATE OF SUMMARY ^a	REPORT CONTROL SYMBOL DD-DR&E(AR)636	
3. DATE PREV SUMRY	4. KIND OF SUMMARY	5. SUMMARY SCTY ^a	6. WORK SECURITY ^a	7. REGRADING ^a	8A. DISSEM INSTR ^a	8B. SPECIFIC DATA - CONTRACTOR ACCESS ^a	9. LEVEL OF SUM A. WORK UNIT
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B. CONTRIBUTING							
C. OTHER WORK		CDOG 114(f)					
11. TITLE (Precede with Security Classification Code) ^a							
(U) Infectious Hepatitis							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS ^a							
002600 Biology 003500 Clinical Medicine							
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
69 07		CONT		DA		C. In-House	
17. CONTRACT/GRANT				18. RESOURCES ESTIMATE		19. PROFESSIONAL MAN YRS	
A. DATES/EFFECTIVE: NA				PRECEDING		B. FUNDS (in thousands)	
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C. TYPE:				CURRENT		74 1.5 35	
D. KIND OF AWARD:				F. CUM. AMT.			
19. RESPONSIBLE DOD ORGANIZATION				20. PERFORMING ORGANIZATION			
NAME: ^a Walter Reed Army Institute of Research				NAME: ^a Walter Reed Army Institute of Research			
ADDRESS: ^a Washington, D.C. 20012				ADDRESS: ^a Division of Medicine Washington, D.C. 20012			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)			
NAME: Buescher, COL E. L.				NAME: ^a Conrad, COL M. E.			
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21. GENERAL USE				SOCIAL SECURITY / ACCOUNT NUMBER [REDACTED]			
Foreign Intelligence Not Considered				ASSOCIATE INVESTIGATORS			
				NAME: Ginsberg, MAJ A. L.			
				NAME:			
22. KEYWORDS (Precede EACH with Security Classification Code)							
(U) Liver Disease; (U) Hepatitis; (U) Australia antigen							
23. TECHNICAL OBJECTIVE, ^a 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
<p>23. (U) Viral hepatitis has been a major cause of morbidity in military populations and a significant hazard in the military blood program. Investigations have been undertaken related to the etiology, epidemiology, and prevention of this group of diseases.</p> <p>24. (U) Methods for the detection of Australia antigen and antibody were evaluated. Studies are in progress to ascertain if hyperimmune gamma globulin prevents transfusional hepatitis B.</p> <p>25. (U) 72 07 - 73 06. Sensitive methods for subtyping Australia antigen and detecting Australia antibody were developed. Significant increases in the prevalence of both HBAG and HBAB were observed in soldiers following military service and particularly after assignment in Asia. HBAG was shown to be a potential hazard in commercial laboratory control serum from multiple manufacturers. A study is in progress among cardiac surgery patients who receive multiple transfusions to ascertain if gamma globulin containing a high titer of HBAB is useful in the prevention of hepatitis B. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 72 - 30 Jun 73.</p>							

^a Available to contractors upon originator's approval.

DD FORM 1498
1 MAR 68

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AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE

PII Redacted

Project 3A062110A822 MILITARY INTERNAL MEDICINE

Task 00, Military Internal Medicine

Work Unit 126, Infectious Hepatitis

Investigators

Principal: Marcel E. Conrad, COL, MC

Associate: Allen L. Ginsberg, MAJ, MC

Description.

A double blind study was initiated to determine whether prophylactic injections of gamma globulin with a high titer for Australia antibody (HBAb) are useful in the prevention of transfusional hepatitis. This study is being performed in collaboration with the National Heart and Lung Institute of HEW and with Letterman General Hospital.

New methods of testing serum specimens for Australia antigen (HBAG) and HBAb were evaluated to ascertain if they were more reliable and practical for use in blood banks. These methods were used to quantify the prevalence of HBAG and HBAb in various troop populations and in biologic specimens.

Biological specimens obtained in volunteer studies of infectious hepatitis at Illinois State Penitentiary in collaboration with Dr. Joseph D. Boggs of Northwestern University are maintained for distribution to investigators attempting to isolate the etiologic agent of hepatitis, develop antibody tests to identify patients with the disease, and develop an animal model of the disease that would obviate the need for human studies.

Results.

In 1946 Grossman et al reported that the parenteral administration of gamma globulin significantly diminished the incidence of transfusional hepatitis. Subsequently these data were confirmed by some investigators and challenged by others. The discovery of Australia antigen (HBAG) permitted better identification of patients with hepatitis B and differentiation of these patients from those with hepatitis A. There is little doubt from a number of clinical studies that injections of immune serum gamma globulin provide protection against the occurrence of hepatitis A. Recent information from our laboratory and others suggests that serum gamma globulin

with a significant HBAb titer may prevent the occurrence of clinical HBAG positive serum hepatitis. These data provide evidence that HBAG positive hepatitis can be prevented by gamma globulin prophylaxis when either soldiers are infected by the nonparenteral route or children are exposed to a small dose of hepatitis B. This information does not provide definitive information regarding the capability of gamma globulin to prevent transfusional hepatitis in which the dose of virus may be larger than in either the experimental studies or following nonparenteral exposure. Aliquots of the gamma globulin used in four transfusion studies were obtained; two of these studies showed significant protection by gamma globulin whereas the others did not. HBAb was titered in aliquots of the gamma globulin used in these four studies both in our laboratory and in two other laboratories, and no significant difference was found between the lots which were protective and those which were not. These data can be interpreted in several ways. Interpretations include: (1) Australia antibody (HBAb) is not the protective factor in gamma globulin, and we should search for another antibody such as that against Dane particles; (2) There was a significant incidence of hepatitis A in the studies in which a protective effect of gamma globulin was demonstrated; (3) methods for testing for HBAb are crude and of insufficient sensitivity; (4) different methods of preparing gamma globulin may produce changes in the antibody protective effect disproportionate to in vitro observations; and (5) Different periods of storage (3 to 10 years) and variable storage procedures (4°C to -15°C) may sufficiently alter antibody titers so that the recent HBAb testing does not reflect the HBAb titers in the gamma globulin at the time the human studies were performed. Since the available information is inconclusive regarding the efficacy of gamma globulin for the prophylactic prevention of transfusional hepatitis, we initiated a new study to determine if gamma globulin with a high HBAb titer provides protection against transfusional hepatitis in collaboration with the National Institutes of Health and Letterman General Hospital.

During recent years there has been a high incidence of hepatitis among patients hospitalized at Walter Reed Army Medical Center undergoing cardiac bypass surgery. Estimates of the incidence of hepatitis in this group based upon the detection of elevated transaminase determinations three months after surgery were 20% of patients. It was believed that this was caused by the requirement for use of large volumes of blood and blood products from multiple donors in these patients during surgery. Since August 1972 all volunteers who were undergoing cardiac bypass surgery received a 10 ml injection of either high titer HBAb gamma

globulin, conventional gamma globulin, or an albumin placebo solution. These injections are administered double blind under code. The blood is drawn from the volunteers before gamma globulin injection weekly after surgery while the patient is hospitalized, and three and six months after surgery. The blood specimen is tested for HBAG, HBAb, SGOT, SGPT, and serum bilirubin determinations. In addition, a history is obtained from each patient at intervals after surgery. All blood used for transfusion is tested by radioimmune assay for HBAG and HBAb. It is estimated that a minimum of 300 patients will be required for completion of the above study. The biologic materials used in this study include a high titer HBAb lot of gamma globulin prepared by the Massachusetts State Laboratories and currently used under NHLI contract in several national studies; a lot of gamma globulin used in 60,000 soldiers in Korea; and a placebo solution used in 40,000 U.S. soldiers in Korea without known complications. All solutions have been tested in accordance with USP and FDA regulations.

During FY 73, 86 cardiac patients were included in this study from Walter Reed Army Medical Center. During March 1973, Letterman General Hospital became a collaborator in the study and had entered 16 patients undergoing cardiac surgery. There have been no known adverse reactions to the administration of either the gamma globulin or placebo solutions. At Walter Reed Army Medical Center, one-fourth of patients have received blood which is HBAG positive by radioimmune assay but negative by counterimmunoelectrophoresis in the blood bank. A 90% followup at three months has been achieved by mail and telephonic followup; noncompliance is largely in children undergoing cardiac surgery. Presently, 40% of patients with three to four months followup have abnormal transaminase determinations in blood specimens obtained 90 to 120 days after surgery. The finding of HBAG is unusual even in patients who have received HBAG positive blood transfusions. Despite the high incidence of elevated transaminase determinations, only two patients have developed overt icteric hepatitis requiring hospitalization. It is anticipated that it will take approximately three years in order to complete this study.

Attempts were made to improve and develop methods for the detection of HBAG that would be more useful in blood banking. The currently available method (counterimmunoelectrophoresis) which is used in blood banks is relatively insensitive. A proposed and recently FDA-approved solid phase radioimmunoassay method for the detection of HBAG has been shown to be more sensitive but requires 24 hours for the performance of the test. Since the majority of the time required for testing is the

double incubation of specimens, we increased the incubation temperatures from room temperature to 45°C and were able to develop a three-hour test. This shorter test was as sensitive as the 24-hour test. Serial dilutions of Ab and Ay subtypes of HBAG were tested simultaneously by both methods. At each dilution both the counts per minute as well as the ratio of counts per minute to the mean of negative controls were higher with the shorter test than with the overnight test, and HBAG detectability was comparable with both tests when specimens obtained from soldiers returning from Vietnam were evaluated. It is believed that this shorter test will be of value for blood banking needs unless a simpler, shorter, and less expensive test can be devised in the interim.

The solid phase radioimmune assay test was modified so it could be used to subtype serum specimens containing HBAG as either ay or ad. The radioimmunoassay system permitted the correct identification of 24 coded specimens that had been previously subtyped by agar gel diffusion. In the series of 52 sera from soldiers with HBAG positive hepatitis, only 7 (13%) could be subtyped by agar gel diffusion, whereas 33 (64%) were subtyped by radioimmunoassay. Twenty-eight (54%) were ad and five (10%) were ay. Thus, solid phase radioimmunoassay methodology provides a means of subtyping many HBAG positive specimens that cannot be subtyped by other methods.

A solid phase radioimmunoassay method was developed to detect HBAb. This method detects specimens which contain antibody against both ay or ad subtypes of HBAG, and seems more sensitive than other laboratory methods. Examination of serum specimens from military recruits showed that 5% had HBAb whereas HBAb was found in the serum of 14% of soldiers arriving in Korea and 25% of soldiers leaving Korea after one year of overseas service. Quantification of the HBAb in lots of gamma globulin that have been used in experimental studies to protect soldiers from icteric serum hepatitis showed no correlation between HBAb titer and the efficacy of the lots of the standard human gamma globulin.

Many commercial firms provide serum control specimens for use in the standardization of routine laboratory tests. We randomly selected ten of these commercially available serum pools and tested them for HBAG by immunoosmoelectrophoresis and radioimmunoassay. Although none was positive by immunoosmoelectrophoresis, nine of the ten specimens contained HBAG as detected by radioimmunoassay. Each of the positive specimens could be inhibited by prior incubation of the test specimen with HBAb. The use of human serum control specimens containing HBAG represents a laboratory hazard that could be avoided and should be insisted upon in specifications.

Conclusions and Recommendations.

The prophylactic gamma globulin study using a high titer preparation containing HBAb will take a minimum of three years for completion. An effort is being made to bring other military and civilian institutions into the study in order to reduce the time required to obtain a meaningful conclusion. There is a requirement for the development and availability of appropriate animal facilities for test programs that would be useful in the development of active and passive immunization for both hepatitis A and B. Further studies of newer methods of testing for HBAG are needed to develop a sensitive, reliable, and useful method for blood banks. Maintenance of a bank of infectious materials for hepatitis A is required to supply investigators attempting to culture the virus and develop serological test systems that will detect patients with the disease.

Project 3AJ62110A822 MILITARY INTERNAL MEDICINE

Task 00, Military Internal Medicine

Work Unit 126, Infectious Hepatitis

Literature Cited.

Publications:

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PROJECT 3A062110A823
MILITARY PSYCHIATRY

Task 00
Military Psychiatry

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL	
				DA OA 6470	73 07 01	DD-DR&F(AK)63A	
3. DATE PREV SUMMARY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8A. DISSEM INSTR	8B. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
72 07 01	D. Change	U	U	NA	NL	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	A. WORK UNIT
10. NO. CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER			
A. PRIMARY	62110A	3A062110A823	00	030			
B. CONTRIBUTING							
C. CONTRIBUTING	CD OG 114(f)						
11. TITLE (Provide with Security Classification Code)							
(U) Military Psychiatry							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS							
003500 Clinical Medicine 013400 Psychology							
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
54 09		CONT		DA		C. In-House	
17. CONTRACT GRANT NA				18. RESOURCES ESTIMATE		19. PROFESSIONAL MAN YRS	
A. DATES/EFFECTIVE				PREVIOUS		B. FUNDS (in thousands)	
B. NUMBER				FISCAL YEAR		73	
C. TYPE				CURRENT		14	
D. KIND OF AWARD				74		16	
E. CUM. AMT.						400	
19. RESPONSIBLE OOD ORGANIZATION				20. PERFORMING ORGANIZATION			
NAME: Walter Reed Army Institute of Research				NAME: Walter Reed Army Institute of Research			
ADDRESS: Washington, D. C. 20012				ADDRESS: Washington, D. C. 20012			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)			
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21. GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER			
Foreign Intelligence Not Considered				ASSOCIATE INVESTIGATORS			
				NAME: Bardill, MAJ D. R.			
				NAME: Nace, MAJ E. P.			
22. KEYWORDS (Provide with Security Classification Code)							
(U) Psychiatric Treatment; (U) Military Adjustment; (U) Psychiatric Illness;							
(U) Stress Performance; Deviant Behavior							
23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
23. (U) The mission of this unit is to identify psychologic, sociologic, organizational and physiologic factors which predispose the soldier to perform ineffectively or develop psychiatric, psychosomatic disease or drug addiction, and to develop appropriate preventive and treatment techniques.							
24. (U) The research methods of psychology, sociology, clinical psychiatry, anthropology, social work, and biochemistry are used to identify and modify factors that contribute to ineffective military performance.							
25. (U) 72 07 - 73 06 The relationship between hypnotizability and the response to brief psychotherapy has been assessed in thirty-two psychiatric outpatients who received brief psychotherapy and had a post-therapy evaluation. Hypnotizability was assessed by standardized techniques. Data analysis is in progress concerning pre- and post-therapy measures of complaints and functioning. Pilot data collection concerning families of adolescents referred to a Child Guidance Service has been completed. These data are being used to define more precise measurement procedures for further work. The influence of social variables upon communications between soldiers of different races at an Army medical facility is under study. Utilizing the Racial Perceptions Inventory with a novel set of instructional variables, data are being collected concerning the ability of an individual soldier to imagine himself in the opposite racial role. An initial evaluation of the utility of role reversal as a technique of behavioral modification is in progress. A follow-up study of more than 100 Vietnam returnees is nearing completion; the extent of drug abuse and other adaptational problems in this population are being evaluated. For technical report, see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 72-30 Jun 73.							

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Project 3A062110A823 MILITARY PSYCHIATRY

Task 00 Military Psychiatry

Work Unit 030 Military Psychiatry

Investigators.

Principal: COL Harry C. Holloway, MC
Associate: LTC James L. Collins, MC; MAJ Donald R. Bardill, MSC; MAJ Edgar P. Nace, MC; MAJ Rodney V. Burbach, MC; CPT Larry H. Ingraham, MSC

Description

Four primary areas were emphasized within the Department of Psychiatry. One area of research was the study of 101 drug using soldiers and 44 mental hygiene controls. Another area of emphasis was a study of the families of adolescents referred for care at a Child Guidance Service. Increasing preliminary emphasis has been placed on studies of race perception inventories and race relations training. Finally, selected data from a brief study of the delivery of Mental Health Services have been analyzed and a report submitted.

Progress

1. Interview Study of 101 Drug-using Soldiers and 44 Mental Hygiene Controls.

The analysis of data from an interview study of 101 drug-using soldiers and 44 mental hygiene controls has been completed.

This study involved soldiers who first became addicted to heroin while serving in Vietnam. Two groups of these soldiers were compared: the first group voluntarily sought help from medical facilities for problems related to opiate dependency after they returned to CONUS; the second group had not voluntarily sought assistance for opiate dependency, but had been involuntarily detected by urine screen as they departed Vietnam, and were sent to CONUS treatment facilities. Distinct differences existed between these two groups as the former had (1) a greater degree of civilian anti-social behavior, (2) more frequent psychiatric contacts while in the service, and (3) more extensive history of drug abuse.

Based on the two groups of addicted Vietnam returnees described above, a prognostic assessment of treatment potential for the opiate

dependent Vietnam returnee was made. This was done by comparing the Vietnam returnees with civilian addicts as reported in the literature on variables which have consistently reflected favorable or poor prognoses. Generally, the Vietnam returnees more closely resemble the civilian addicts who had a favorable outcome following treatment. The addicted Vietnam returnees who were detected by urine screen techniques had a more favorable prognosis than did the other group of addicted Vietnam returnees, although both groups resembled more closely successfully treated rather than chronically addicted civilian addicts.

A detailed analysis of drug usage patterns was conducted in order to determine whether or not potential heroin addicts could be distinguished from non-addicted but multi-drug using peers. On the basis of age of onset of drug use, drug of initiation, number of drugs used, order of use, rapidity of progression from one drug to another and other selected usage variables, no meaningful differences between the addicted and non-addicted drug users could be determined. The non-addicts actually exceeded the addicts on many of the drug usage variables. Differences between these two groups do occur with regard to drug use after narcotics are introduced. Those who were to become opiate dependent rapidly became addicted, while the other group nearly as quickly dropped opiates from their drug-using repertoire. Case illustrations are presented and implications for the so-called "stepping stone" hypothesis are discussed.

A study was conducted in order to determine whether soldiers who presented medical facilities with a problem of drug abuse could be distinguished from "non-drug problem presenting" mental hygiene patients. A step-wise linear discriminant analysis was utilized for this problem, and utilized only data available from civilian history and the first five months of military duty, "i.e., prior to being defined as a drug user or other type of patient." On the bases of both historical and drug usage variables, it is possible to distinguish between these two groups of psychiatric patients as early as the first five months of military duty. These results may be of value in any attempts at primary prevention.

2. Hypnosis and Brief Psychotherapy.

A study assessing the relationship between hypnotizability and the response to brief psychotherapy has been completed. Each of the thirty-two psychiatric outpatients was evaluated, underwent brief psychotherapy and had a post-therapy evaluation. Hypnotizability was assessed prior to the beginning of therapy by standardized, objective techniques. Selected pre- and post-therapy measures of patient com-

plaints and functioning were obtained. The data have been tabulated and are presently undergoing statistical analysis. The returns from a six-month follow-up are also being currently collected and entered into the analysis. This project was carried out with the assistance of several psychiatry residents at Walter Reed General Hospital, and a report for the Resident Research Seminar is being prepared.

3. The Family and Adolescent Dysfunctioning.

The pilot data collection phase of a study of the families of adolescents referred for care at a Child Guidance Service has been completed. Data gained from the pilot phase are being used to refine the study procedure and to develop new and refine old data gathering instruments. Preliminary work has suggested additional family areas for investigation.

4. Race Relations, Racial Perceptions Inventory and Race Relations Training.

Preliminary work continues in a study of effects of race relations training on WRAMC personnel. Additional studies to further develop race relations inventories continue to progress.

The Racial Perceptions Inventory (R.P.I.) will be administered to 7 biracial groups of 10 military personnel, to be recruited on a voluntary basis in July 1973. The objectives will be to determine whether the revised R.P.I. will show previously obtained differences in black and white perceptions and to evaluate the influence of instructional factors upon these differences.

5. Project Home.

Project HOME is a longitudinal study of a little more than 100 Vietnam veterans. Drug use, and certain other parameters of life style are being followed. All subjects cooperated in a rather lengthy initial interview. Those subjects who were remaining in the Army (about 1/2) were re-interviewed every two months. Those subjects who have separated from the Army are being contacted by means of mailed questionnaires, about one year after the initial contact. The study contains approximately equal numbers of each of the following four categories; (1) urine negative, non-drug users, (2) urine negative drug users, (3) urine positive recreational drug users, and (4) urine positive drug dependent users. Urine test results refer to the DEROS screen from Vietnam.

At the present time, all initial interviews have been completed. The follow-up interviews are complete for those persons who remained in the Army. In progress is the mailing of the follow-up questionnaires to those persons who separated from the Army. Preliminary pencil-and-paper tabulations have been done on the data from the initial questionnaires.

Project 3A062110A823 MILITARY PSYCHIATRY

Task 00 Military Psychiatry

Work Unit 030 Military Psychiatry

Literature Cited.

Publications:

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION ^a	2. DATE OF SUMMARY ^a	REPORT CONTROL SYMBOL DD-DR&E(AR)636	
3. DATE PREV SUMMARY	4. KIND OF SUMMARY	5. SUMMARY SCTY ^a	6. WORK SECURITY ^a	7. REGRADING ^a	8. ORIGIN INSTN ^a	9. SPECIFIC DATA- CONTRACTOR ACCESS ^a	10. LEVEL OF SUM A. WORK UNIT
72 07 01	D. Change	U	U	NA	NI	<input type="checkbox"/> YES <input type="checkbox"/> NO	
11. NO./CODES: ^a	PROGRAM ELEMENT	PROJECT NUMBER		TASK AREA NUMBER		WORK UNIT NUMBER	
a. PRIMARY	62110A	3A0-2110A823		00		031	
b. CONTRIBUTING							
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11. TITLE (Precede with Security Classification Code) ^a (U) Military Performance and Stress; Factors Leading to Decrements of Performance and Disease. (09)							
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e. KIND OF AWARD:	f. CUM. AMT.		74				
19. RESPONSIBLE DOD ORGANIZATION				20. PERFORMING ORGANIZATION			
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21. GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER: [REDACTED]			
Foreign Intelligence not Considered				ASSOCIATE INVESTIGATORS			
				NAME: Jennings, CPT J.R.			
				NAME: Thorne, D. Ph.D. DA			
22. KEYWORDS (Precede EACH with Security Classification Code) (U) Electrophysiology; (U) Biorhythms; (U) Psychophysiology; (U) Operant Conditioning; (U) Stress; (U) Performance; (U) Human Volunteer							
23. TECHNICAL OBJECTIVE: ^a 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
<p>23. (U) Stressful environments, physiological conditions and performance demands likely to produce significant deterioration in the accomplishment of a soldier's mission are studied. The behavioral and physiological functions that contribute to deteriorated performance are identified and therapeutic and prophylactic strategies are developed.</p> <p>24. (U) Using psychophysiological and operant methodology, time series analysis, and computer-based control and analysis techniques, behavioral and physiological events are isolated, analyzed, and controlled. Endogenous and exogenous factors contributing to behavioral and physiological rhythmicity and performance levels are studied under specified normal and stressful conditions.</p> <p>25. (U) 72 07 73 06 Extensive time series analyses including complex demodulation have demonstrated ultradian rhythmicity within psychophysiological activity during a 48-hour sleep deprivation study. Performance follow-up of Officer Candidate School graduates indicates no relationship between OCS ratings and subsequent performance. Maturational and personality variables minimally predicted post OCS performance. Speeded information storage and accompanying autonomic reactions differed between obese and underweight subjects. Autonomic reaction differed as a function of type of information processing and information load. Development of miniature, field adaptable, electrophysiological recording techniques continues. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 JUL 72- 30 JUN 73.</p>							

^a Available to contractors upon originator's approval.

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PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 68 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE.

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Project 3A062110A823 MILITARY PSYCHIATRY

Task 00 Military Psychiatry

Work Unit 031 Military performance and stress: Factors leading to decrements of performance and disease

Investigators.

Principal: Frederick W. Hegge, Ph.D.

Associate: MAJ Malcolm G. Robinson, MC; CPT John R. Jennings, MSC; David Thorne, Ph.D.; CPT John Varni, MSC; 1LT Charles Wood, MSC; Paul Kasper, B.S.; James Struthers, B.A.; Jeanne Stringfellow, B.A.

Description

The elucidation of the biological substrates of stress and performance decrements is important to both military psychiatry and to the performance of normal military missions. The basic research strategy of this work unit is psychophysiological in nature, i.e., concurrent measures of behavioral processes and physiological activity are made. Special attention is paid to stressors having their origin in continuous performance requirements, sleep deprivation, and temporal disorientation. Due recognition is given to the fact that performance is not a unitary construct, but a continuum of human activity ranging from simple motor behavior to the most complex cognitive activity. Research is directed at the experimental delineation of interactions between stressors and complex performances that are functional analogues of militarily relevant activities. These include vigilance, the integration of multiple sources of information, and decision processes. When necessary for scientific clarity, complex performances are analyzed in terms of more basic processes involving sensorimotor, attentional, and mnemonic components.

Progress

1. Sleep Loss, Sustained performance, and the Basic Rest-Activity Cycle (BRAC).

A requirement for sustained performance under conditions of stress is not unusual in military settings. The stress levels involved range from those associated with combat to the relatively mild levels associated with sleep deprivation. Performance requirements similarly range from simple motor behavior to the most complex reasoning and decision making. The

literature on stress and sustained performance is confused concerning what performances are affected by what stressors under what conditions. Part of this confusion may arise from the presence of circadian (circa 24 hours) and ultradian (circa 90 minutes) rhythms in the quality of performance.

Rapid Eye Movement (REM) sleep has long been known to exhibit a rhythm having a period ranging from 80 to 120 minutes. Kleitman has proposed that this REM cycle is a manifestation of a fundamental endogenous oscillation of the central nervous system that occurs throughout the day. Serman, Lucas & MacDonald (1972) have recently presented evidence for a REM-related periodicity in the operant behavior of cats, and Kripke (1972) has presented related data on electrophysiological function in humans. The presence of a rhythmic fluctuation in performance and physiology could serve as a confounding variable in studies that rely on data sampled infrequently in time. Performance sampled at a peak in a cycle would appear to be better than performance measured later during a cycle minimum. Thus, opposite conclusions could be drawn if the sampling were shifted slightly in time. We have no reason to believe that the rhythms of different individuals would be precisely coordinated in time. Therefore, at any given sampling time, values for one subject may be high while those for another are low. The operation of any of these factors could easily lead to inflated variance and erroneous conclusions.

A study designed to investigate these factors has been completed and various aspects of the data have been reported at two scientific meetings. Twelve human volunteers attempted to detect movements within a visual display while confined to an isolation chamber for periods potentially as long as 48 hours. Only limited and random breaks for eating and elimination were permitted. The payment for participation increased as time in the chamber increased, but no subject completed a full 48 hour run. The longest run was 44 hours and the shortest was 22 hours. On leaving the chamber, subjects uniformly reported that they had "had it" and could not continue under any circumstances.

Heart rate, rate of observing the display, and rate of signal detection were collected continuously and summarized every fifteen minutes. These data formed time series that were subjected to a complex demodulation analysis. Complex demodulation is a filtering technique whereby the amplitude and phase of selected narrow frequency bands can be estimated for every data point in a time series. It is particularly useful where the series are nonstationary, i.e., the statistical properties of the series change over time. Studies of continuous performance fall into this category.

The major finding of this study was the confirmation of the existence of

a 90-minute cycle in heart rate, observing behavior, and signal detection. Further, the amplitude of this rhythm reliably increased with stress. In the present case the stressors are operationally defined as sleep deprivation and the sustained performance requirement. Subjects refused to continue in the experiment at, or just below the point of maximum rhythm amplitude. In the latter case, subjects had been persuaded to "stay with it just a while longer".

These findings have important implications for the quantification of stress induced performance decrement, for the cross comparison of stressors and their interaction with different performance requirements, and for the explication of individual differences in response to stress. They provide a clear cautionary note to the interpretation of data gathered using traditional point sampling procedures. Finally, the finding of a short-term rhythm in performance suggests an experimental model for the study of potentially critical lapses in the quality of performance continuity. If, as these findings suggest, performance lapses are orderly rather than random, prophylactic strategies can be developed to protect the integrity of critical performances.

2. Measurement Technology for the Assessment of Sustained Performance.

The monitoring of behavioral and physiological processes for extended periods of time places a heavy burden on signal acquisition and processing equipment. The burden is partially due to the large volume of data acquired, e.g., simple summarization of a variable once per minute generates 1440 data points per day. In addition, there are severe requirements for equipment performance reliability and stability. Equipment worn by active subjects must be unobtrusive and non-invasive. Electrodes must adhere and have low noise characteristics in the face of extensive body movement. Amplifiers and recorders must be insensitive to large muscle potential artifacts while retaining high sensitivity for the signal of interest. Monitoring equipment must require an absolute minimum of intervention by investigators for operation checks, recording medium changes, or data readouts. These problems have been approached in two ways during the past year.

The Bioengineering Division of the British Medical Research Council has developed one electrochemical and two magnetic tape recording systems for the continuous monitoring of electrophysiological signals. Informal cooperative arrangements with the 361st Medical Laboratory, USAR, have provided opportunities to evaluate this equipment. Most promising is a four channel analog recorder capable of retaining 24 hours of data on a standard tape cassette. Preliminary tests involving continuous monitoring of EEG before, during and after a transcontinental flight were successful.

Further field tests and operation in the Cardiac Care Unit of the Walter Reed General Hospital are planned.

The second approach involves the in-house development of a fully digital subminiature recorder fabricated from the most advanced large scale integrated circuits. Design work is nearing completion and many of the component parts have been acquired for a prototype unit. In concept, the system involves the acquisition and conditioning of signals followed by their conversion to digital format at the lowest sampling rate consonant with maintenance of the information content of the signal. Digitized signals are then stored in a semiconductor memory for later readout. The virtue of this system is that readout can be accomplished at very high speeds. A full day of data can be made available for processing in seconds. Present magnetic tape systems are limited in time compression capability to approximately 1/120th of the record duration.

Three small, innovative instrumentation devices were developed and written up for publication during the last year: 1) a device for simulating waking and sleeping EEG patterns, 2) a general purpose microvolt test signal generator, and 3) an improved stereotactic instrument used to implant recording electrodes. Similar development of small useful laboratory devices is continuing through the normal operation of our electronics shop.

3. Longitudinal Study of Endocrine Reactions and Stress in Officer Candidate School.

This study has been completed and results have been prepared for publication. As previously described (Kreuz, Rose, & Jennings, 1972), the study was conducted in three phases: 1) acute stress phase during the third week of OCS; 2) baseline stress phase during the 23rd week - a period of minimal stress; and 3) follow-up phase after the candidates had been at their PCS six months. A battery of endocrine measures, interviews, and psychological tests were administered during both phases 1 and 2. The follow-up phase consisted of a telephone interview assessing the former candidate's duty performance.

The major results after complete statistical analyses confirmed impressions reported earlier: 1. Plasma testosterone levels were reduced dramatically during the early, stressful phase of OCS. 2. Endocrine measures, performance scores and personality indices were minimally related if at all. 3. Psychiatric interview assessment showed a moderate (.4) correlation with final rank in OCS. 4. Measures relating to first impression formation by officers and motivational distortion by candidates were

correlated to OCS class standing. 5. OCS class rank did not predict follow-up performance.

Additional analyses sought refined relationships between platoon membership, class standing, stress, and coping scores. The results of these were suggestive, but could not be supported scientifically.

4. Autonomic Correlates of Information Processing.

Autonomic nervous system reactions have been considered primarily as indices of stress. Evidence is emerging (Lacey & Lacey, in press; Hahn, 1973) that suggests, however, that autonomic reactions are involved in relatively nonstressful psychological acts, such as attending to the environment and problem-solving. If these autonomic reactions are an important part of such psychological acts, then interference with the autonomic reactions might lead to a decrement in performance. As stress exerts marked influence on autonomic reactions, autonomic disturbances may be an integral part of the syndrome of stress-related performance decrements.

An investigation of the specific autonomic correlates of various types of information processing has been completed. In addition, the influence of obesity was studied due to a recent suggestion that the information processing capability is related to body weight (Schachter, 1971). The primary dependent variables were cardiac inter-beat-interval (IBI) and task performance although measures of skin conductance, muscle potentials, and affective ratings of the tasks were also collected.

Episodes of shortening and lengthening of the cardiac IBI were associated with different psychological functions. Instances of shortening seemed more closely related to affective involvement or arousal. Lengthening of the IBI seemed a function of the demand for either attention to the environment or active memory processing. Skin conductance related to the arousal dimension, but muscle potentials were not influenced by the experimental variables.

Obese subjects were expected to show enhanced attention toward input and more lengthening of the cardiac IBI than underweight subjects. These expectations were generally borne out when the difficulty of the information processing was low. When difficulty was increased, the obese, in comparison to the underweight, performed less well on the memory tasks and the cardiac IBI was no longer lengthened beyond base level.

Thus, the experiment demonstrated the sensitivity of cardiac IBI to different types of information processing across tasks and across individuals of different body weights.

Further work is planned to validate the relation of cardiac IBI to attention and memory under varying conditions of memory overload and attentional salience. The role of the autonomic reactions in the information processes may be studied by pharmacologically blocking certain autonomic reactions.

5. Statistical Problems Associated with Biological Measures.

The measurement of biological data characteristically takes place over time within an individual. This produces a series of data points which are not statistically independent, and are therefore not readily dealt with by classical statistical techniques. Two approaches to this problem have been made. First, statistical separation of cardiac components via factor analytic techniques has been attempted, but the meaning of the emerging results is still unclear. Second, specialized time series techniques are being developed. Classical time series analyses are not strictly applicable to data, such as cardiac inter-beat-Interval (IBI), which is not a continuous function. Work is now being initiated to the application of point-process time series analyses for the assessment of biorhythms-type data as well as evoked response-type data. These techniques may provide a viable alternative to current measurement techniques.

In addition, certain traditional statistical problems, such as the nature of the distribution of a variable, arise continually within an ongoing research project. Work on the distribution characteristics of cardiac IBI has been prepared for publication. IBI was distributed more normally than instantaneous heart rate. Assessment of the IBI distributions of Viet Nam heroin users is underway.

Project 3A062110A823 MILITARY PSYCHIATRY

Task 00 Military Psychiatry

Work Unit 031 Military performance and stress: Factors leading to decrements of performance and disease

Literature Cited.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1 AGENCY ACCESSION ^a	2 DATE OF SUMMARY ^a	REPORT CONTROL SYMBOL DD-DR&E(AH)636	
3 DATE, PREV SUMMARY	4 KIND OF SUMMARY	5 SUMMARY SCTY ^a	6 WORK SECURITY ^a	7 REGRADING ^a	8A DDB'S INSTR ^a	8B SPECIFIC DATA- CONTRACTOR ACCESS ^a	9 LEVEL OF SJM A. WORK UNIT
72 07 01	H. Termination	U	U	NA	NL	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	
10 NO CODES ^a	PROGRAM ELEMENT	PROJECT NUMBER		TASK AREA NUMBER		WORK UNIT NUMBER	
A. PRIMARY	62110A	3A062110A823		00		032	
B. CONTRIBUTING							
C. CONTRIBUTING	CDOG 114(f)						
11 TITLE (Precede with Security Classification Code) ^a							
(U) Drug Abuse in Military Personnel							
12 SCIENTIFIC AND TECHNOLOGICAL AREAS ^a 002300 Biochemistry; 012900 Physiology; 013400 Psychology; 003500 Clinical Medicine; 016800 Toxicology							
13 START DATE		14 ESTIMATED COMPLETION DATE		15 FUNDING AGENCY		16 PERFORMANCE METHOD	
71 07		72 08		DA		C. In-House	
17 CONTRACT GRANT NA				18 RESOURCES ESTIMATE		A. PROFESSIONAL MAN YRS	
A. DATES/EFFECTIVE				PRECEDING		B. FUNDS (in thousands)	
B. NUMBER ^a				FISCAL YEAR		72 20 600	
C. TYPE				CURRENT		73 100 3000	
D. KIND OF AWARD				73		100 3000	
19 RESPONSIBLE DOD ORGANIZATION				20 PERFORMING ORGANIZATION			
NAME ^a Walter Reed Army Institute of Research				NAME ^a Walter Reed Army Institute of Research			
ADDRESS ^a Washington, D. C. 20012				Division of Neuropsychiatry			
RESPONSIBLE INDIVIDUAL				ADDRESS ^a Washington, D. C. 20012			
NAME ^a Buescher, COL E. L.				PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)			
TELEPHONE 202-576-3551				NAME ^a Holloway, COL H. C.			
				TELEPHONE 202-576-3556			
				SOCIAL SECURITY ACCOUNT NUMBER [REDACTED]			
21 GENERAL USE				ASSOCIATE INVESTIGATORS			
Foreign Intelligence Not Considered				NAME: Angel, LTC C. R.			
				NAME: Lamson, COL T. H. DA			
22 KEYWORDS (Precede EACH with Security Classification Code) (U) Drug Abuse; (U) Epidemiology; (U) Biochemistry; (U) Treatment/Rehabilitation; (U) Prevention; (U) Toxicology							
23 TECHNICAL OBJECTIVE, 24 APPROACH, 25 PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
23. (U) To develop and assess screening techniques for military settings; to determine the extent, prevalence, and incidence of drug abuse by soldiers, to devise methodologies for the identification, treatment and prevention of drug abuse by soldiers, and to assess the effects of drug usage upon military performance.							
24. (U) All systems purported to be useful for screening are evaluated in the laboratory and, if indicated, tested in the field; developmental changes in these systems are made. Studies assessing drug usage are conducted in military populations; intervention programs are evaluated, using epidemiological, social, and psychological methods; effects of drugs upon performance investigated, and the laboratory assessment of the toxicological, biochemical and psychological basis of illicit drug use are examined.							
25. (U) 72 07 - 72 08 Terminated; to be reported under 833 WU 102.							

^aAvailable to contractors upon originator's approval

DD FORM 1498

PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1495A 1 NOV 68 AND 1498 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE

PII Redacted

PROJECT 3A062110A824
IONIZING RADIATION INJURY, PREVENTION AND TREATMENT

Task 00
Ionizing Radiation Injury, Prevention and Treatment

1064

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION ^a	2. DATE OF SUMMARY ^a	REPORT CONTROL SYMBOL DD-DR&E(AR)636	
3. DATE PREV SUMMARY	4. KIND OF SUMMARY	5. SUMMARY SCTY ^a	6. WORK SECURITY ^a	7. REGRADING ^a	8. DES'N INSTR'N	9. SPECIFIC DATA- CONTRACTOR ACCESS	10. LEVEL OF SUM
72 07 01	D. CHANGE	U	U	NA	NL	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	A. WORK UNIT
10. NO./CODES ^a		PROGRAM ELEMENT		PROJECT NUMBER		TASK AREA NUMBER	
a. PRIMARY		621110A		3A062110A824		00 055	
b. CONTRIBUTING							
c. CONTRIBUTING		1212B (21)					
11. TITLE (Precede with Security Classification Code) ^a							
(U) Chemical Protection Against Irradiation (09)							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS ^a							
014000 Radio and Radiation							
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
59 05		CONT		DA		C. In-House	
17. CONTRACT/GRANT				18. RESOURCES ESTIMATE		19. PROFESSIONAL MAN YRS	
a. DATES/EFFECTIVE: NA				PRECEDES		b. FUNDS (in thousands)	
b. NUMBER ^a				FISCAL YEAR		73 6 150	
c. TYPE				CURRENT		74 1.85 130	
d. KIND OF AWARD				e. CUM. AMT.			
19. RESPONSIBLE DOD ORGANIZATION				20. PERFORMING ORGANIZATION			
NAME: Walter Reed Army Institute of Research				NAME: Walter Reed Army Institute of Research			
ADDRESS: Washington, D.C. 20012				Division of Medicinal Chemistry			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)			
NAME: BUESCHER, E.L., COL				NAME: Sweeney, T.R., Ph.D.			
TELEPHONE: 202/576-3551				TELEPHONE: 202/576-3731			
21. GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER			
Foreign Intelligence Not Considered				ASSOCIATE INVESTIGATORS			
				NAME: Rothe, William E., COL DA			
22. KEYWORDS (Precede EACH with Security Classification Code) ^a							
(U) Activity; (U) Chemical; (U) Compound; (U) Dose; (U) Protection; (U) Radiation Injury; (U) Human Volunteers							
23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
<p>23. (U) The objective of this research is to develop a militarily useful pill to protect personnel against lethal effects of ionizing radiation. Such a drug would reduce the initial effect of nuclear radiation as well as provide a margin of safety for personnel operating in a contaminated terrain. An efficient antiradiation compound would also be useful to the Army from a clinical standpoint.</p> <p>24. (U) Approach to the objectives is through accepted drug development protocols. Synthesis and testing of potential agents is being carried out. Test results are analyzed for structure activity relationships and fed back into the synthesis program. Promising compounds are carried forward to testing in large animals and the pharmacology of these compounds investigated. In addition, chronic toxicity studies, dose reduction factor studies and drug antagonism studies are being carried out.</p> <p>25. (U) 72 07 - 73 06 The synthesis effort was phased out; emphasis was on the synthesis of important disulfides desired for biological testing. Screening of new compounds was resumed after a 10-month delay for purchase and installation of a cesium-(3) radiation source. Large animal studies were continued with a shift in emphasis from parenteral studies in dogs to oral studies in monkeys. While aminoalkylphosphorothioates continue to be the most active class of drugs, promising new thiazolidines and disulfides have been identified. A Phase I human tolerance test has been completed on WR 2721. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 July 72 - 30 June 73.</p>							

^aAvailable to contractors upon originator's approval

DD FORM 1498

PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A - NOV 72 AND 1498B - 1 MAR 74 FOR ARMY USE ARE OBSOLETE

1004 Z

PII Redacted

Project 3A062110A824 IONIZING RADIATION INJURY, PREVENTION AND TREATMENT

Task 00 Ionizing Radiation Injury, Prevention and Treatment

Work Unit 055 Chemical Protection Against Radiation

Investigators.

Principal: Thomas R. Sweeney, Ph.D.

Associates: COL William E. Rothe, VC; 1LT Blair T. Atherton, MSC;
Miss Marie Grenan; 1LT T. Scott Griffin, OrdC;
Melvin H. Heiffer, Ph.D.; LTC Kenneth E. Kinnamon, VC;
Daniel L. Klayman, Ph.D.; LTC Peter S. Loizeaux, VC;
Robert S. Rozman, Ph.D.; Mrs. June A. Schafer;
2LT Thomas S. Woods, CmlC

Problem

The possible use of nuclear weapons in future warfare in both strategic and tactical situations poses a significant hazard for troops which must operate in the resulting radioactive environment. Measures to decrease the radiation hazard and thereby increase the soldier's effectiveness are required.

Background

Almost 25 years ago a new field of research was opened when it was found that certain chemicals administered before radiation with x-rays brought about a substantial reduction of radiation injury in rats. The possibility was thus made evident of the development of a drug which could provide for the soldier a significant degree of protection from various radiation hazards associated with nuclear warfare. As a consequence the program to develop useful antiradiation drugs was established at WRAIR in 1959. The goal was the development of an orally effective drug with a dose reduction factor (DRF) of 3 when taken 24 hours or more prior to radiation exposure and having a wide margin of safety. Steady progress has been made. Seven drugs have been effective enough to have received an investigational exemption for the development of a new drug application. Four of the seven are still being actively pursued. The leading single drug developed has a dose reduction factor of 2.3 when administered parenterally to mice and a duration of effect on the order of 5 hours. Interest in the development of an antiradiation drug has decreased since the signing of the Nuclear Test Ban Treaty. The project is currently in a period of an orderly phase-out of several years duration.

Approach

The approach to the development of an antiradiation agent has followed the medicinal chemical research patterns used successfully by pharmaceutical companies and their development of new drugs. Based upon

feedback from the biological test system and the biochemical literature, structure-activity relationships are established which served as a basis for the synthesis of more effective compounds. Those compounds considered particularly effective are resynthesized in larger quantities for testing in dogs and/or monkeys. Those compounds that survived the large animal studies are then subjected to toxicity, pharmacokinetic, and a pharmacodynamic studies in order to determine the suitability of the compound for clinical studies and to compile the data necessary to claim an investigational exemption.

Results and Discussion

General progress during FY-73 reflected the effects of the phase-out of the Drug Development Program. The rate of the introduction of new compounds was reduced because of the diminished synthesis program. In addition testing has been delayed because of necessary changes in the physical facilities.

Additional studies (COL Ray Olsen, MC, Principle Investigator) on WR-2721 have been carried out. The drug has been administered orally to human volunteers up to 5 grams per day. The data are in the process of being analyzed.

Chemistry: Contract Synthesis Program. The three chemical synthesis contracts that were active during FY-73 were in a phase-out period. Two of the contracts that will enter FY-74 will expire relatively soon after the new year. The Preparations Laboratory continued to synthesize compounds in larger amounts for large animal studies.

In view of the imminent termination of the synthesis program, the decision was made that the thrust of the synthesis should be in the area of disulfides which were needed to complete several series of active compounds. The disulfides were particularly indicated because of their stability and likelihood of high activity. In addition there was synthesized a number of the active aminoalkylaminoethylphosphorothioate class, a number of compounds with novel sulfur-blocking group, and three difficultly synthesized cyclobutane aminothiols; the latter are of considerable theoretical interest because of the stereochemistry involved. A total of 83 new compounds were submitted by the synthesis laboratories and four submitted in large quantities from the Preparations Laboratories.

Chemistry: Organic Chemistry Laboratories. Investigations have been continued on the synthesis of new and effective antiradiation agents.

The sodium borohydride reduction of organic thiosulfates has until recently eluded efforts to produce thiols directly in both protic and non-protic solvents. The reaction has produced for the most part organic disulfides which have resisted cleavage in situ due, apparently,

to the presence of borohydride by-products. It has now been found that when the reaction is performed in dimethylsulfoxide at 80-100°, an acceptable yield of thiol is obtained. Studies which are anticipated to lead to improve yields of thiols and purer products are still in progress.

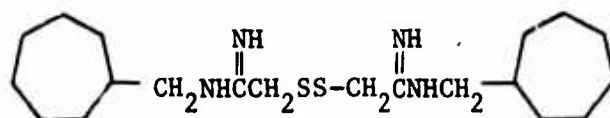
In the investigation of the effect of thiobenzoate ion on organic thiosulfates, which was completed this year, it was found that the course of the reaction is influenced by the degree of substitution of any amino group present in the substrate molecule. This finding is in harmony with the earlier observation that aminoalkylthiosulfuric acids react with inorganic sulfide ion in a manner dependent upon the nature of the amino group present. Compounds derived from this study are undergoing evaluation for antiradiation activity.

A method has been developed for the rapid detection of organic polysulfides in the presence of disulfides. The method depends upon the selective cleavage of polysulfides with sodium borohydride in aqueous or aqueous ethanol solution followed by an acidification which results in a copious evolution of hydrogen sulfide. This technique has been utilized in confirming compound identity in instances of questionable labeling of containers of di- and polysulfides.

A number of potential antiradiation agents, active intraperitoneal¹, has been resynthesized in larger quantities for oral testing.

Biology: Rodent Testing Program. No radiation studies were performed in mice for the first 8 months of FY-73 as a consequence of the removal of an obsolete cobalt 60 small animal irradiator and the purchase, installation, loading and calibration of a gamma cell 40 cesium-137 source. During the final four months of the year, drug screening was resumed but only on a reduced basis because of the remodeling of the animal room with the resulting reduction of animal holding facilities. A total of 220 tests were performed on 118 drugs. Additional N-substituted amidines were tested to complete the studies on the structure-activity relationship in the active amidine series. Additional analogs of the highly active WR-2721 and WR 3689 are being studied. The eight months suspension in testing has resulted in a buildup of a backlog of 500 compounds for testing, of which 350 are new drugs.

Biology: Large Animal Testing Program. Emphasis during the past year has been placed upon oral administration of drugs, utilizing the rhesus monkey as the model. Eight compounds were tested, three of which showed activity. WR 151,331, a disulfide, protected 2 of 5 monkeys when administered orally at 75 mg/kg two hours prior to irradiation.



WR 151,331

Oral studies have continued to be discouraging; the compounds tested do not approach the protective activity that is obtained following intravenous administration. It seems possible that the high gastric acidity of the monkey may result in the breakdown of certain classes of radio-protective drugs, e.g. phosphorothioates, although the use of a variety of buffers in conjunction with test drugs was tried without success. WR-3689, a highly active phosphorothioate when administered intravenously, has been prepared in enteric-coated tablets but not yet evaluated.

Three drugs WR 108,503, WR 168,643, and WR 50,909 were tested by intravenous administration to dogs; none showed remarkable activity. The total number of antiradiation drug evaluations was significantly reduced as a result of reactor down-time for refueling and equipment calibration.

A system for visually monitoring the radiation dose in the exposure room of the Diamond Ordnance Radiation Facility TRIGA reactor was developed. The system uses small on line ionization chambers constructed of magnesium and polystyrene and filled with CO₂ connected via coaxial cables to a control room amplifier and strip chart recorder. The system enables the reactor operator to precisely control the total radiation dose delivered to the experiment.

Biology: Liver Perfusion Studies. In general, hepatic neoplasia is still considered to be an inoperable condition. The classic treatment is to perfuse the liver with nitrogen mustard by intra-arterial catheterization. However, it has been shown that malignant neoplasms growing in the liver tend to acquire an exclusively arterial blood supply, regardless of the route by which tumor emboli reach the liver. In contrast the normal parenchyma receive blood primarily via the portal circulation.

One can be selective in the perfusion of the liver due to the two distinctive circulatory patterns of the neoplastic organ. Mustargen introduced via the arterial (hepatic) route should go primarily to the neoplasms and hepatic tissue immediately adjacent to them. Introduction of a mustargen antagonist through the venous (inferior mesenteric) system has shown to protect the normal parenchyma in pilot studies previously conducted in dogs. It is reasonable to assume that mustargen doses sufficient to eliminate hepatomas, although ordinarily lethal when injected into the systemic circulation can be successfully counter-

acted by concurrent administration of a suitable nitrogen mustard antagonist.

Baseline studies have been run in normal Macaca mulatta monkeys. In three monkeys of the same species which had confirmed hepatomas (monkeys with hepatomas supplied by Dr. Richard H. Adamson, National Cancer Institute) the dose regimens employed seemed to enhance rather than impede tumor growth.

Biology: Therapy of Radiation Injury by Hematopoietic Tissue Grafting: A Study of Graft Elimination. This work is being accomplished in collaboration with the Armed Forces Radiobiology Research Institute, Bethesda, Maryland.

Radiation-exposed individuals that would die from damage to the hematopoietic system may be saved by grafting bone marrow from a suitable donor. However, unless the donor and recipient are identical with respect to histocompatible antigens, individuals that overcome the radiation death may succumb to a serious immunological malady. Any measure that reduces the immunologic reaction increases the chances for successful therapy. This reduced immunologic reaction may be accomplished by either increasing the graft-host compatibility or eliminating the foreign graft after it is no longer required for survival. The former approach is the one most commonly employed and is the one of choice when the subject being treated has an abnormal hematopoietic system. However, when the subject had, before the radiation exposure, a normal hematopoietic system the latter approach, i.e. eliminating the foreign graft, may be preferable. Data presented here and the evidence reported by others is reason to believe that marrow graft elimination is feasible and may be preferable to the more popular method of promoting graft-host compatibility.

Initial experiments are being conducted to establish the relationship between the time after radiation and the minimum size of a C57B1 6 mouse marrow graft which will permit CBA mice exposed to a lethal dose of whole-body radiation to survive the radiation crisis. After administration of 20-, 40-, 100-, and 300 x 10⁶ viable cells on days 1, 2, 5, 7, 10, and 14 after radiation exposure it has been shown that 1) bone marrow administration at 12 or 14 days, regardless of cell level, does not prevent a radiation death; 2) cell doses of 300 x 10⁶ are excessive and cause death earlier than in control mice; 3) the maximum time allowable after radiation exposure in which to prevent a radiation death may be as great as 10 days; 4) the optimum cell dose is 40- to 100 x 10⁶ cells. Studies are continuing.

Conclusions

Significant protection against the effects of ionizing irradiation can be provided by drugs if they are administered prior to radiation exposure. Species variability, toxicity, and the variability in response with route of administration for various classes of compounds

will continue to pose serious testing difficulties. Parenteral administration is the most suitable route for screening purposes to determine if a drug has radioprotective properties. Oral administration poses a variety of difficulties not encountered with the parenteral route but is the route of choice for the soldier in the field and therefore should be vigorously pursued. The synthesis of increasingly active antiradiation drugs, as demonstrated by DRF and duration of effect, supports the approach adopted. The original criteria, that is, oral administration, a DRF of 3, a 24 hour duration of action and a wide margin of safety are realistic goals.

Recommendations

The orderly phase out of the antiradiation drug development program that is now underway should be continued until all synthesized compounds have been adequately tested. A preparations laboratory should be maintained in order to provide a resynthesis and purification capability for interesting compounds uncovered during the final testing period. The synthesis contracts should be brought to an orderly termination. Emphasis in the biology should be placed on oral administration of drugs and on techniques to prevent premature destruction and enhance absorption in the gastrointestinal tract.

Project 3A062110A824 IONIZING RADIATION INJURY, PREVENTION AND TREATMENT

Task 00 Ionizing Radiation Injury, Prevention and Treatment

Work Unit 055 Chemical Protection Against Radiation

Literature Cited.

References:

1. Klayman, D.L. and Woods, T.S.; The effect of Thiobenzoate Anion on Organic Thiosulfates, International J. Sulfur Chem., in press (1973).
2. Klayman, D.L., Griffin, T.S., and Woods, T.S.; The Use of Sodium Borohydride to Distinguish Organic Polysulfides from Disulfides, International J. of Sulfur Chem., in press (1973).

Publication:

1. Klayman, D.L. and Griffin, T.S.; Reaction of Selenium with Sodium Borohydride in Protic Solvents. A Facile Method for the Introduction of Selenium into Organic Molecules, J. Amer. Chem. Soc., 95, 197 (1973).

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION ^a	2. DATE OF SUMMARY ^a	REPORT CONTROL SYMBOL DD-DR&E(AR)636	
3. DATE PREV. SUMRY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8A. DMRN INSTRN	8B. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
72 07 01	D. Change	U	S	3	NL	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	A. WORK UNIT
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A. PRIMARY	62110	3A062110A824		00		057	
B. CONTRIBUTING							
C. CONTRIBUTING	CD0G1212B(21)						
11. TITLE (Precede with security Classification Code)							
(U) Biological Effects and Hazards of Microwave Radiation							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS ^a							
013400 Psychology; 016208 Stress Physiology; 005700 Electronic & Electrical Engineering							
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
71 07		Cont		DA		C In House	
17. CONTRACT/GRANT				18. RESOURCES ESTIMATE		19. PROFESSIONAL MAN YRS	
A. DATES/EFFECTIVE		B. EXPIRATION		PRELIMINARY		C. FUNDS (in thousands)	
NA				FISCAL YEAR		73	
B. NUMBER		C. TYPE		CURRENT		4	
A. KIND OF AWARD		D. AMOUNT				380	
		E. CUM. AMT.		74		5	
						550	
20. RESPONSIBLE DOD ORGANIZATION				21. PERFORMING ORGANIZATION			
NAME: Walter Reed Army Institute of Research				NAME: Walter Reed Army Institute of Research			
ADDRESS: Washington, DC 20012				ADDRESS: Division of Neuropsychiatry Washington, DC 20012			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)			
NAME: Buescher, COL E.L.				NAME: Sharp, J.C., Ph.D.			
TELEPHONE: 202-576-3551				TELEPHONE: 202-576-5126			
22. GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER: [REDACTED]			
Foreign Intelligence considered				ASSOCIATE INVESTIGATORS			
				NAME: Grove, H.M.			
				NAME: Hawkins, T.D. DA			
23. KEYWORDS (Precede EACH with Security Classification Code)							
(U) Microwave Hazards; (U) Nonionizing Radiation; (U) Dosimetry; (U) Behavioral Effects; (U) Neurophysiology; (U) Military Medicine							
24. TECHNICAL OBJECTIVE, 25. APPROACH, 26. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
<p>23. (U) To establish meaningful criteria to delimit human operations in an electromagnetic environment to support maximum combat effectiveness at minimum personnel risk from the environment. Delineate the interaction of radiofrequency and microwave radiation (100 MHz to 100 GHz) with biological systems. Survey and evaluate all known methods and techniques of microwave dosimetry and develop improved techniques and instrumentation where appropriate and necessary.</p> <p>24. (U) Investigate each major organ system and biological process where there is reason to believe microwave effects may occur at reasonable power intensities. Where indicated, determine the military significance of the effects and the measures necessary to obviate them. A data bank of the world literature on the biological effects and hazards of electromagnetic radiation is to be maintained. Initial scientific efforts will use evaluative methods from experimental psychology, electrophysiology and ophthalmology. Exposure parameters will be chosen for relevance to Army radiating equipment and operational requirements.</p> <p>25. (U) 72 07 - 73 06. During the reporting periods effort has been concentrated on the behavioral assessment of the effects of power levels up to 150 mW/sq. cm at various wavelengths; a series of lethality studies in rodents have been completed; and a replication of an experiment involving turnover of brain serotonin completed. With work stoppage as an end point a definite effect of the wavelength of radiation has been observed with a maximum effect at 1700 MHz. A survey of the literature on microwave cataractogenesis has been conducted and a series of studies initiated. A major conference was organized dealing with the biological effects of microwaves, the proceedings are in the process of publication. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 July 72 - 30 June 73.</p>							

DD FORM 1498
1 MAR 68

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PII Redacted

Project 3A062110A824 IONIZING RADIATION INJURY, PREVENTION AND
TREATMENT

Task 00 Ionizing Radiation Injury, Prevention and Treatment

Work Unit 057 Biological effects and hazards of microwave radiation

Investigators.

Principal: Joseph C. Sharp, Ph.D.

Associate: H. Mark Grove, M.Sc.; Sandra H. Githens, B.S.;
T. Daryl Hawkins, M.A.; MAJ Stuart E. Hirsch, MC;
MAJ Lawrence E. Larsen, MC; John F. Schrot, M.A.;
Peter Brown, B.S.

During FY 73, the second year of funding, several studies have been completed and a relatively large and comprehensive program has been initiated. This Annual Report summarizes the salient points of progress in five general areas.

I. BEHAVIORAL BIOLOGY STUDIES OF MICROWAVE EFFECTS.

In the past year, substantial gains have been obtained in our attempts to develop a systematic framework within which the orderly study of behavioral and biological effects of microwave irradiation can be conducted. Three major studies have been completed, along with several pilot investigations, which have led to observations of the potentially important consequences of exposure to microwaves.

Lethal effects of 3.0 GHz exposure of rats.

In a first study of lethality, an elliptical focusing antenna was employed to provide high density levels. One hundred and seventy-four male rats were exposed for durations of 30 to 240 seconds at a frequency of 3.0 GHz (i.e., 10 cm wavelength). At each exposure duration, the percent mortality increased with increases in incident energy density (power x duration). The LD₅₀ proved to be a monotonic function of exposure duration, at least within the ranges investigated. This relationship seems to have considerable generality since similar functions were later found in behavioral studies and in a lethality study utilizing the free-field, anechoic exposure space. Within a relatively narrow range (180 to 210 grams), it was found that body weight is an important determiner of lethality, i.e., heavier animals are more susceptible than the lighter animals.

Lethal effects of 2.45 GHz exposure of rats.

Utilizing a free-field technique, and observing rats with closed circuit television, it was possible to determine the relationship of

seizures and death to exposure variables. As in the 3.0 GHz study, a monotonic relation was found as a function of energy density. It was also found that the higher the power level, the shorter the time to convulsion. Within-subject comparisons demonstrated that death followed convulsions by about 60 seconds and was independent of the exposure duration required to produce the convulsion. The utility of further investigations into this hyperthermic convulsion may be dependent upon the need for, and interest in, hyperthermia research and/or pharmacological intervention into "fever" states.

Frequency dependent effects on rat performance.

A study was completed which assessed the performance of rats in a far-field exposure environment as a function of both frequency (wavelength) and power density. The rats were trained to perform a simple task requiring ten lever presses to produce a food pellet; they could obtain their daily food ration in a 15-minute session. At each exposure frequency (0.75, 1.7, 2.45 and 3.0 GHz), the animals were exposed to five different power levels (25, 50, 75, 100, and 150 mW/cm²). The highest power levels resulted in the termination of performance. The effect of varying frequency, however, was more complex in that a "U-shaped" function was generated. It appears that 1.7 GHz has the greatest effect upon performance when power levels are kept constant. Both the higher and lower frequencies had progressively less impact upon the performance than did 1.7 GHz. It is extremely important that the generality of this frequency sensitivity be confirmed. This generality should be investigated across both dependent variables (lethality, convulsions, behavioral assessments, and physiological variables) and independent variables (species, body weights and geometry, wavelength and energy levels).

Species comparisons of lethality.

Now that considerable information has been developed on the rat in both lethality and behavioral studies, it is necessary to develop comparisons with other, different species. Preliminary observations indicate the mouse is more vulnerable to 2.54 GHz exposure than is the laboratory rat. At a power density of 200 mW/cm², the time to convulsion and time to death were found to be approximately 50-percent shorter in the mouse than in the rat. By contrast, at the same power densities, the New Zealand rabbit is less sensitive than the rat, with times to convulsion and death approximately twice those of the rat. These species differences suggest that body size is an important determiner of the animals' response to exposure. Different physiological mechanisms, or efficiency of similar mechanisms in different species, may be as important as the size of the organism. Work on the interrelationship between such variables and wavelength clearly must be pursued to a logical conclusion.

II. CENTRAL NERVOUS SYSTEM ELECTROPHYSIOLOGY.

Since it is a central hypothesis of this laboratory that electrophysiological effects coincident with microwave exposure may be a result of thermoregulatory dysfunction or inadequacy, considerable effort has been devoted to the development of a system to measure brain temperatures during microwave exposure. Such a system, coupled with a means to measure electrophysiological activity, will allow us to follow the time-course of thermoregulatory responses, electrical activity and local brain temperature gradients. After fabricating many different probe designs and testing them for both radio-frequency (RF) and electrical decoupling, it was found that a probe using microcircuitry techniques was very promising. The final probe has the following characteristics: The thermistor lead "wires" consist of balanced lines 2 μm wide, 2000 Å thick, and 2 μm apart, this to minimize loop area and thereby power extraction. Long term RF heating of the mount, due to microwave dipole currents is suppressed through the action of current limiting flip-chip resistors and series resistors. This microwave integrated circuit (MIC) is connected to an external full bridge and detector by means of high-impedance, conductive monofilament. By using infra-red thermography it was demonstrated that long-term heating due to RF power absorption was eliminated. This finding showed that the combination of microcircuitry, current-limiting series resistance and thermal isolation makes possible continuous temperature measurements in animals in the presence of microwave radiation.

The development of an electrode which is decoupled from the RF fields is technically and conceptually simpler than the development of a temperature sensing probe. Such electrodes have now been made and are in the process of being validated and calibrated. After these necessary and time-consuming preliminary steps are completed, the probes and electrodes will be implanted in rabbits and the animals then exposed to various microwave environments. All of the peripheral and ancillary equipment and programs have been developed and are ready to be used.

III. OCULAR EFFECTS.

During the reporting period, the study of microwave ocular effects has continued on two major fronts: The clinical examination of occupationally exposed personnel and laboratory studies of rabbits exposed to various wavelengths.

Clinical investigation.

Various military installations which employ relatively large numbers of individuals who work with microwave generating equipment are periodically visited by a team of clinical and research ophthalmologists. A complete ophthalmic examination is made of these

individuals as well as a cohort of non-occupationally exposed people. To date, some 2,000 examinations have been made by the visiting team. While some lens pathologies have been observed, the distribution of these abnormalities between the exposed and non-exposed workers has tended to be nearly the same. There may be some slight tendency for the older (>50 years old) workers, who are occupationally at risk, to have a slightly higher incidence and prevalence of lens changes. This project is continuing, and the data are not yet fully collected or analyzed.

Laboratory microwave ocular research on rabbits.

Work has been completed during the past year on the establishment of parameters necessary for the production of lenticular opacification development in albino New Zealand rabbits. In an initial study, single exposures to 3.0 GHz CW irradiation at varying power densities from 25-500 mW/cm², utilizing the elliptical focusing antenna were performed with follow-up of animals for one year. In no case was the single sub-lethal exposure noted to cause lenticular opacifications for this period of follow-up and with the power levels employed.

Microwave cataracts have been produced in this strain of rabbits, however, by utilizing the focusing antenna at power levels greater than 400 mW/cm², following eleven daily 15-minute exposures. Serial slit-lamp biomicroscopic photographs have been taken to document the morphology and progression of the lenticular changes. Selected animals have been sacrificed at various stages of opacification and both light and electron microscopic histologic examination of the tissues performed. This will provide for a more complete understanding of relations between slit-lamp appearance of posterior subcapsular iridescence and the microscopic morphology.

Utilizing the far-field, anechoic chambers, and operating at either 2.45 or 1.7 GHz, it has been demonstrated that whole-body exposures to 50 mW/cm² for two hours are lethal. A study was designed in which nine rabbits were exposed to 25 mW/cm² for two hours daily for a total of 27 days, 5 days per week. Serial observations of the eyes of each rabbit were performed after each exposure and, to date, the animals have been followed for two months post exposure. No lenticular changes have been noted in the animals still alive. Four animals, however, died of respiratory infections.

Presently, work is being completed which will provide a dose-response curve for lenticular changes in rabbits exposed daily to 3.0 GHz. Similar data will be generated at different frequencies (i.e., 1.7 and 2.45 GHz, at least) and, later, in a different species (e.g., dogs).

IV. CELLULAR RESPONSES TO 2.45 GHz EXPOSURE.

Two types of studies have been designed and completed which were a first attempt to identify the effects of relatively low-level microwave exposure ($\leq 33 \text{ mW/cm}^2$) on cell activity. The first series of studies started with the hypothesis that cellular activity, as reflected by H^3 -thymidine uptake, may be altered in a microwave field. Using a highly proliferative tissue, intestinal mucosa of rats, and measuring H^3 -thymidine uptake per mg of tissue, there was found to be an increase in uptake following exposure to 16 and 8 mW/cm^2 when the whole animal was exposed in a multi-modal cavity. In a free-space situation the increased uptake was found at 16 mW/cm^2 , but not at 8 mW/cm^2 . These findings were interpreted to cast doubt on the dosimetry of the multi-modal cavity vis-a-vis the far-field dosimetry. The findings are at power levels slightly below and above the current acceptable level of 10 mW/cm^2 ; the observation of an effect at this power level should in no way be construed to have an impact on hazard determinations. By use of radioautographic techniques, we have demonstrated that the tritium is in the cells of the mucosa and not in the smooth muscles or fascia which strongly suggested an increase in mitotic activity.

Because of the importance of a mechanism which might be useful in stimulating cellular mitotic activity, as well as the exquisite experimental controls necessary to demonstrate the effect, it was felt important to look directly at mitotic figures and count their occurrence. Since mitotic activity had been shown to vary markedly over a 24-hour period with a maxima during mid-morning for rodents, a study using mice entrained to a day/night cycle was designed. The animals were exposed, and later sacrificed, either in the morning or evening. The corneal epithelium was removed and stained to facilitate mitotic spindle counting. The results of this study clearly demonstrated the rhythmic nature of the mitotic activity and showed the time of maximal sensitivity to microwave exposure to be in the evening. That is, more spindles per 1000 were found 3 to 6 hours after the exposure if the exposure occurred during the time when there would normally be little such activity. The implications of this observation for the design of future experiments are important since it is obvious that to "control" for rhythmic phenomena by rigidly adhering to a fixed time-of-day for making observations may obscure important biological events. This becomes especially true when the dependent variables are subtle and apparently ephemeral.

The next series of studies in this area will deal with other tissue systems and will attempt to integrate the finding that there may be wavelength specific effects. The frequency of 2.45 GHz was initially chosen because it is the frequency of nearly all "radar" ovens, and the ability to directly go from a multi-modal to a

far-field environment was theoretically important. The finding that performance decrements show a frequency-dependent effect, with the maximum being at 1.7 GHz, demands an investigation of lethality, time to convulsions, and cellular activity at this frequency as well.

V. LITERATURE DATA BASE.

Efforts have continued to build an automated data base of the world literature related to the biological effects of electromagnetic radiation. This work is under contract to Mead Technology Laboratories. During FY 73, approximately 300 papers have been acquired which have been published during the period January 1971 through April 1973. The base is currently being jointly evaluated by agencies active in this research area, namely the Army, the U.S. Environmental Protection Agency, and the U.S. Public Health Service, Bureau of Radiological Health. Depending upon the results of this evaluation and upon current analysis of the legal problems concerned with the inclusions of copyrighted material in the base, a decision will be made toward the end of FY 74 as to the future of this operation.

PROJECT 3A663713D829
MALARIA PROPHYLAXIS

Task 00
Malaria Investigations

1079

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION ^a	2. DATE OF SUMMARY ^a	REPORT CONTROL SYMBOL DD-DR&E(AR)636	
3. DATE PREV SUMRY	4. KIND CP SUMMARY	5. SUMMARY SCTY ^a	6. WORK SECURITY ^a	7. REGRADING ^a	8. DOWN INSTN ^a	9. SPECIFIC DATA - CONTRACTOR ACCESS	10. LEVEL OF SUM A. WORK UNIT
72 07 01	H. TERMINATION	U	U	NA	NL	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	
10. NO./CODES ^a	PROGRAM ELEMENT	PROJECT NUMBER		TASK AREA NUMBER	WORK UNIT NUMBER		
6. PRIMARY	63713A	3A663713D829		00	106		
7. CONTRIBUTING							
8. CONTRIBUTING	CDOG 114 (F)						
11. TITLE (Precede with Security Classification Code) ^a							
(U) Antigenic Fractionation, Serology of Malaria							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS ^a							
002600 Biology							
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
65 07		72 08		DA		C. IN-House	
17. CONTRACT/GRANT NA				18. RESOURCES ESTIMATE		19. PROFESSIONAL MAN YRS	
a. DATES/EFFECTIVE:				b. PRESENTS			
c. NUMBER:				FISCAL YEAR		d. FUNDS (in thousands)	
e. TYPE:				72		3 100	
f. KIND OF AWARD:				73		3 100	
g. CUM. AMT.							
20. RESPONSIBLE DOD ORGANIZATION				21. PERFORMING ORGANIZATION			
NAME: Walter Reed Army Institute of Research				NAME: Walter Reed Army Institute of Research			
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				SOCIAL SECURITY ACCOUNT NUMBER: [REDACTED]			
22. GENERAL USE				ASSOCIATE INVESTIGATORS			
Foreign Intelligence Not Considered				NAME: MOON, A. P. DA			
				NAME:			
23. KEYWORDS (Precede EACH with Security Classification Code) (U) Malaria; (U) Plasmodium; (U) Immunity; (U) Erythrophagocytosis; (U) Autoimmunity; (U) Diagnosis							
24. TECHNICAL OBJECTIVE, 25. APPROACH, 26. PROGRESS (Furnish individual paragraphs identified by number. Precede last of each with Security Classification Code.)							
23 (U) To isolate, purify and characterize various antigens from malarial parasites in order to improve diagnostic specificity and to induce immunoprophylaxis or immuno-suppression in military personnel.							
24 (U) Separate parasite proteins by physical and chemical means. Determine the presence and activity of metabolic antigens in the plasma of acutely infected animals and human patients. Analyze the fractionated proteins by both classical and new methods.							
25 (U) 72 07 - 73 06 Work unit is being combined with work unit number 129, line item 3A663713D829.							

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1079 a

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION ^a	2. DATE OF SUMMARY ^a	REPORT CONTROL SYMBOL DD-DR&E(AR)636	
3. DATE PREV SUMMARY ^a	4. KIND OF SUMMARY	5. SUMMARY SCTY ^a	6. WORK SECURITY ^a	7. REGRADING ^a	8. DES'N INSTR'N	9. SPECIFIC DATA - CONTRACTOR ACCESS ^a	10. LEVEL OF SUM ^a
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13. CONTRIBUTING							
14. CONTRIBUTING	CDOG 11A(F)						
15. TITLE (Precede with Security Classification Code) ^a							
(U) Biochemical Effects and Mechanism of Action of Chemotherapeutic Agents							
16. SCIENTIFIC AND TECHNOLOGICAL AREAS ^a							
002300 Biochemistry 002900 Biology							
17. START DATE		18. ESTIMATED COMPLETION DATE		19. FUNDING AGENCY		20. PERFORMANCE METHOD	
64 07		Cont		DA		C. In-House	
21. CONTRACT/GRANT				22. RESOURCES ESTIMATE		23. PROFESSIONAL MAN YRS	
A. DATES/EFFECTIVE: NA				PRECEDING		B. FUNDS (In thousands)	
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C. TYPE:				CURRENCY		13	
D. KIND OF AWARD:				74		5	
E. AMOUNT:						245	
F. CUM. AMT.						200	
24. RESPONSIBLE DOD ORGANIZATION				25. PERFORMING ORGANIZATION			
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26. GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER: [REDACTED]			
Foreign Intelligence Not Considered				ASSOCIATE INVESTIGATORS			
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				NAME: Siu, P.M.L., Ph.D., Kazyak, L.B.S. DA			
27. KEYWORDS (Precede EACH with Security Classification Code) ^a							
(U) Malaria Chemoprophylaxis							
(U) Malaria Chemotherapy; (U) Drug Action; (U) Drug Analysis; (U) Plasmodium							
28. TECHNICAL OBJECTIVE, 29. APPROACH, 30. PROGRESS (Punish individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
23. (U) To define toxicity, mechanisms of action, and pharmacokinetics of experimental antimalarial drugs.							
24. (U) 1) To use malaria in animal models to define interactions of chemotherapeutic agents with host and parasite. 2) To develop new analytical techniques to study levels of experimental drugs in biological materials.							
25. (U) 72 07 - 73 06 Incubation of P. berghei with iron and chloroquine prior to inoculation into mice delayed onset of parasitemia which was proportional to the concentration of iron indicating a direct effect of iron on the parasite to potentiate chloroquine. Dapsone given orally to rats causes adrenal dystrophy which is not reversible upon withdrawal of the drug. Dapsone produces reversibly lowered blood levels of thyroxin and TSH. P. berghei infection in hamsters causes I-131 retention associated with reduced I-131 excretion. A gas liquid chromatographic method using electron capture detection was developed for determination of WR 30,090 in biological materials. Clinical serum samples were analyzed quantitatively to determine blood levels for correlation with the clinical observations. WR 30,090 is concentrated mainly in the plasma fraction with only very low levels of drug in the erythrocytes. For technical report, see Walter Reed Army Institute of Research Annual Progress Report, 1 July-30 June 73.							

^a Available to contractors upon originator's approval.

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Project 3A663713D829 MALARIA PROPHYLAXIS

Task 00 Malaria Investigations

Work Unit 108 Biochemical effects and mechanism of action of chemotherapeutic agents

Investigators.

Principal: LTC Douglas J. Beach, MSC

Associate: Nesbitt D. Brown, B.S.; Larissa deBaare, M.D.;
LTC Gale E. Demaree, MSC; John W. Diggs, Ph.D.;
Seymour Garson, Ph.D.; Leo Kazyak, B.S.;
Robert T. Lofberg, Ph.D.; Benjamin Mehlman, M.S.;
Robert C. Permisohn, B.S.; Nell R. Pendleton, B.S.;
Patrick M. L. Siu, Ph.D.; H. Kenneth Sleeman, Ph.D.;
SP4 Jaroslaw Slusarenko, B.S.

The objective of this work unit is to apply biochemical principles for the definition of the toxicity, mechanisms of action and pharmacokinetics of experimental antimalarial drugs in animal models and to apply this knowledge to the study of clinical materials.

Specifically the following studies were accomplished during this reporting period.

1. Effects of ferrous iron on the action of chloroquine.
2. Dapsone toxicity in rats.
3. Experimental malaria in the golden hamster.
4. Analysis of WR 30090.
1. Effects of ferrous iron on the action of chloroquine.

Previous reports from this laboratory described the administration of ferrous iron to potentiate the effect of chloroquine to suppress P. berghei in mice. Experiments were conducted to separate the effects of iron on the host mechanisms from the effects of iron on the parasite. In these latter experiments, parasitized erythrocytes were incubated with mixtures of iron and chloroquine prior to injection into the mice. There was a delay in the development of parasitemia which was related to the concentration of ferrous iron in the incubation mixture. These findings indicate that the action of iron to potentiate the effects of chloroquine to suppress malaria in mice are due to the action of iron directly on the parasite. Attempts to extend similar studies of the interaction of iron and chloroquine to P. falciparum in Aotus trivirgatus failed for technical reasons.

2. Dapsone toxicity in rats.

Previous reports from this laboratory described the adrenal and thyroid abnormalities associated with ingestion of dapsone by rats. The present studies were conducted to describe the mechanisms of these endocrine aberrations.

Purified dapsone was administered orally to groups of rats in increasing dosage and increasing duration. Administration of dapsone was withdrawn from subgroups of each above group for varying periods of time. This treatment revealed a gradation of the extent of adrenal morphologic changes that were related to the total dosage of dapsone administered. There was a concomitant decrease in plasma corticosterone levels. Withdrawal of dapsone for up to 30 days showed only partial reversibility of the adrenal toxicity of the drug. No morphologic changes were noted in thyroid tissues in these experiments; but plasma thyroxine levels were decreased and plasma thyroid stimulating hormone levels were increased in a dose-related manner. These thyroid-related abnormalities were completely reversible within 30 days following withdrawal of dapsone. These findings indicate that the mechanisms of adrenal and thyroid toxicity of dapsone are different and may not share a common biochemical pathway.

3. Experimental malaria in the golden hamster.

Studies designed to describe the biochemical parameters of experimental *P. berghei* infections in the golden hamster were concluded with the description of the effects of malaria infection on the handling of parenteral I-131 iodide by the hamster. The infected animals retained I-131 in proportion to the severity of the disease; the levels of I-131 accumulation in the thyroid increased in proportion to the severity of the disease. Total serum I-131 increased in malaria but serum PBI levels decreased and malaria-infected animals excreted less I-131 than non-infected animals. Histopathologic studies of the thyroid tissues from these animals are in progress by the Division of Pathology. No other mechanism studies are in progress or contemplated. Observation of long-term surviving hamsters following *P. berghei* infections revealed an immune-like response. Parasitemia and signs of malaria began between 3-4 weeks post-infection and blood smears became negative for parasites at about 6 weeks; persistence of infection could be demonstrated through 7 weeks by subinoculation into mice. Eradication of parasites was suggested in several cases where subinoculation into mice failed to elicit an infection. Hamsters were also noted to be refractory to homologous superinfection. These findings suggest that the hamster has merit as a model to study certain aspects of acquired immunity to malaria.

4. WR 30090 analysis.

To support clinical evaluations of WR 30090, a dependable procedure for the determination of the antimalarial in red cells and plasma was required. A procedure was developed whereby the drug could be recovered adequately and reproducibly with a single extraction of biological fluid. At least 90% of the available antimalarial can be recovered. Available antimalarial is that amount of the drug that can be consistently released from the total protein-bound portion. Data acquired to date indicate that less than 40% of the total protein-bound drug is released and therefore available for analysis.

The procedure involves denaturing the specimen with methanol to release the protein-bound drug followed by chloroform extraction. The solvent was evaporated, re-dissolved in dimethylformamide and derivatized with n,o-bis-(trimethylsilyl)-acetamide (BSA). An aliquot was chromatographed on an OV-1 column in a gas chromatograph equipped with a Ni-63 electron capture detector.

The major part of the WR 30090 is found in the plasma. With those specimens exhibiting higher concentrations in the plasma (above 0.2 mg WR 30090/liter), small amounts of the drug appear in the red cells. The presence of the drug in serum has not yet been determined, but these tests are in progress.

Project 3A663713D829 MALARIA PROPHYLAXIS

Task 00 Malaria Investigations

Work Unit 108 Biochemical effects and mechanism of action of chemotherapeutic agents

Literature Cited.

Publications:

1. Sleeman, H. K., Diggs, J. W., and Angel, C. R.: The effects of 4,4,-diaminodiphenylsulfone on selected physiological parameters. Abstract, 164th Annual Meeting, American Chemical Society, 1972.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION ^a	2. DATE OF SUMMARY ^a	REPORT CONTROL SYMBOL	
				DA OB 6471	73 07 01	DD-DR&E(AR)636	
3. DATE PREV SUMMARY	4. KIND OF SUMMARY	5. SUMMARY SCTY ^a	6. WORK SECURITY ^a	7. REGRADING ^a	8A. DRG'S INSTR ^a	8B. SPECIFIC DATA - CONTRACTOR ACCESS ^a	9. LEVEL OF SUB A. WORK UNIT
72 07 01	D. Change	U	U	NA	NL	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	
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a. PRIMARY	63713A	3A66371 3D829		00		112	
b. CONTRIBUTING							
c. X-REFERENCE	CDOG 114(f)						
11. TITLE (Precede with Security Classification Code)							
(U) Field Studies on Drug Resistant Malaria							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS ^a							
002600 Biology; 003500 Clinical Medicine; 010100 Microbiology							
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
69 07		CONT		DA		C. In-House	
17. CONTRACT/GRAANT				18. RESOURCES ESTIMATE		19. PROFESSIONAL MAN YRS	
a. DATES/EFFECTIVE: NA				PRECEDING		b. FUNDS (in thousands)	
b. NUMBER: ^a				FISCAL YEAR		6.3	
c. TYPE:				CURRENT		260	
d. KIND OF AWARD:				74		6.0	
e. CUM. AMT.						330	
19. RESPONSIBLE DOD ORGANIZATION				20. PERFORMING ORGANIZATION			
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				SOCIAL SECURITY ACCOUNT NUMBER: [REDACTED]			
21. GENERAL USE				ASSOCIATE INVESTIGATORS			
Foreign Intelligence Not Considered				NAME: Segal, MAJ H. E.			
				NAME: Pearlman, MAJ E. J. DA			
22. KEYWORDS (Precede EACH with Security Classification Code) (U)Drug Resistant Malaria; (U)Chloroquine; (U)Human Volunteer; (U)In vitro models; (U)Tetracycline; (U)Glucose-6-Phosphate Dehydrogenase							
23. TECHNICAL OBJECTIVE, ^a 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
<p>23. (U) To determine the clinical picture of malaria in hospital populations in Thailand. To define entomologic variables which influence the prevalence of chloroquine resistant falciparum malaria. To evaluate antimalarial agents as prophylactics against malaria. To determine the antimalarial efficacy of drugs both old and new.</p> <p>24. (U) Surveys were conducted of the patients with malaria in hospitals in Central and Southeastern Thailand. Longitudinal entomologic studies of malaria transmission were conducted in Central Thailand. Studies of prophylaxis with antimalarial drugs were conducted on rural populations. U.S. Army investigational antimalarial drugs were compared with standard drugs for the treatment of falciparum malaria.</p> <p>25. (U) 72 07 - 73 06 Malaria was highly prevalent year round at a hospital in South-eastern Thailand but was less prevalent and was chiefly confined to the rainy season at a hospital in Central Thailand. During the dry season the life cycle of <i>A. balabacensis</i> is maintained in the forest as opposed to village sites. Chloroquine treatment did not enhance the infectivity of chloroquine resistant strains of <i>P. falciparum</i>. Both WR33063 and WR30090 proved to be effective antimalarial drugs and less toxic than quinine. A combination of pyrimethamine and DDS proved to be an effective prophylactic against malaria.</p> <p>For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 72-30 Jun 73.</p>							

^aAvailable to contractors upon originator's approval

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PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A 1 NOV 68 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE

1085

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Project 3A663713D829 MALARIA PROPHYLAXIS

Task 00, Malaria Investigations

Work Unit 112, Field studies on drug resistant malaria

Investigators.

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Associate: MG Pung Phintuyothin, MC, RTA (Ret.); LTC Bunharn Laixuthai, MC, RTA; CPT Bruce A. Harrison, MSC; CPT John S. Jewell, MSC; CPT Roland N. Wilkinson, MSC; Sanong Kosakal, M.D.; Chalard Tirabutana, M.D.; Y.M. Huang, Ph. D.; John E. Scanlon, Ph. D.; SFC Ben F. Castaneda; SP4 Carl W. Ames; Sumroeng Bumnetphund; Dumrong Charoendhum; Suvath Hanchalay; Vichentr Mettaprakong; Amporn Nanakorn; E.L. Peyton; Prachar Pooyindee; Rampa Rattanarithikul; Sunthorn Sirithanakarn; Withoon Thiemanun; Kosol Vetbutanapibul.

1. Hospital Survey of Malaria in Prachinburi, Central Thailand.

A survey was performed to determine how much malaria is seen at the Prachinburi Hospital, the severity of the disease, and what drugs are used for treatment. The hospital keeps records of the number of patients with malaria and these are presented. Therapeutic studies were performed at the hospital between July 1972 and January 1973. (See reports by MAJ Segal). The survey was conducted on all patients admitted with malaria to the adult wards. The pediatric ward was not included. The SMRL team performed quantitative parasite counts on the majority of patients who were admitted.

Between 1968 and 1971 the hospital diagnosed malaria in between 500 and 900 individuals annually. Deaths from malaria ranged from 27 to 58 (Figure 1). It is not clear whether all these diagnoses were confirmed by microscopy. The seasonal variation in the malaria rate is shown in Figure 2. Peaks occurred in July

TABLE 1a.

PRACHINBURI HOSPITAL 1971. MONTHLY INCIDENCE OF MALARIA
AND AREAS WHERE PATIENTS LIVED

	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Total	Percent
Malarial Cases	43	23	28	31	47	98	136	103	81	117	95	60	862	13.37
P. falciparum	43	23	28	31	47	98	134	100	78	115	95	60	852	98.84
P. vivax	-	-	-	-	-	-	2	3	3	2	-	-	10	1.16
Deaths	2	1	2	1	2	8	10	6	7	8	6	5	58	6.73

TABLE 1b.

LOCATION	TOTAL	%
Kabin Buri	224	25.99
Other Province	187	21.69
Sa Kaeo	125	14.50
Si Mahaphot	124	14.39
Prachinburi City	93	10.80
Prachantakham	67	7.77
Wat Thananakhon	29	3.36
Aranya Prathet	9	1.04
Ban Sang	3	0.35
Ta Phraya	1	0.12
Incidence of complications		
Cerebral	17	1.97
Jaundice	15	1.74
Recrudescence	29	3.36

TABLE 2.

PRACHINBURI HOSPITAL SURVEY NOVEMBER 1972
SEXUAL DISTRIBUTION OF COUNTS OF P. FALCIPARUM

Parasite Count ¹	Total Patients	Male	Female	% Female
< 1,000	13	7	6	46%
1,000-100,000	44	30	14	32%
> 100,000	19	14	5	26%
TOTAL	76	51	25	

¹ Asexual parasites per cmm

TABLE 3.

PRACHINBURI HOSPITAL NOVEMBER 1972. FREQUENCY OF
ADMINISTRATION OF EACH TYPE OF ANTIMALARIAL THERAPY

Intravenous Quinine	Fansidar	Oral Chloro-quine	Primaquine	Oral Quinine	Bactrim	Tetracycline
60	44	34	24	6	2	2

Table 4.

MALARIA DIAGNOSES IN THE SMRL OUTPATIENT CLINIC,
PRACHINBURI HOSPITAL 1972

MONTH	PATIENTS SCREENED*	FALCIPARUM	VIVAX
SEPTEMBER	63	17	2
OCTOBER	74	23	6
NOVEMBER	97	41	7
DECEMBER	88	36	8

* These figures do not include patients examined for the first time after admission to a ward.

Fig. 1.

PRACHINBURI HOSPITAL. ANNUAL CASES OF
MALARIA AND DEATHS. 1968 - 1971

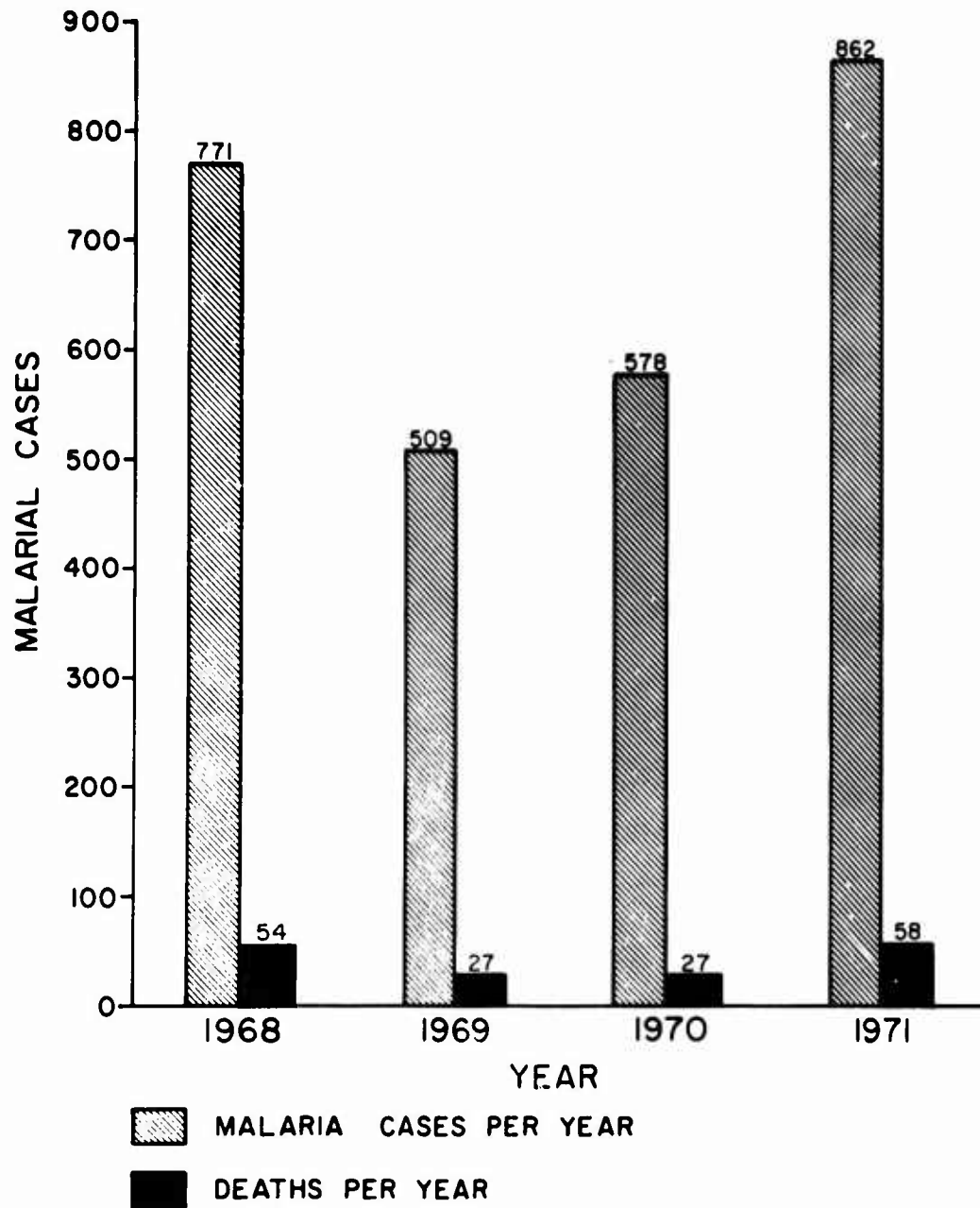


Fig. 2.
PRACHINBURI HOSPITAL
MONTHLY DIAGNOSES OF MALARIA 1968-1971

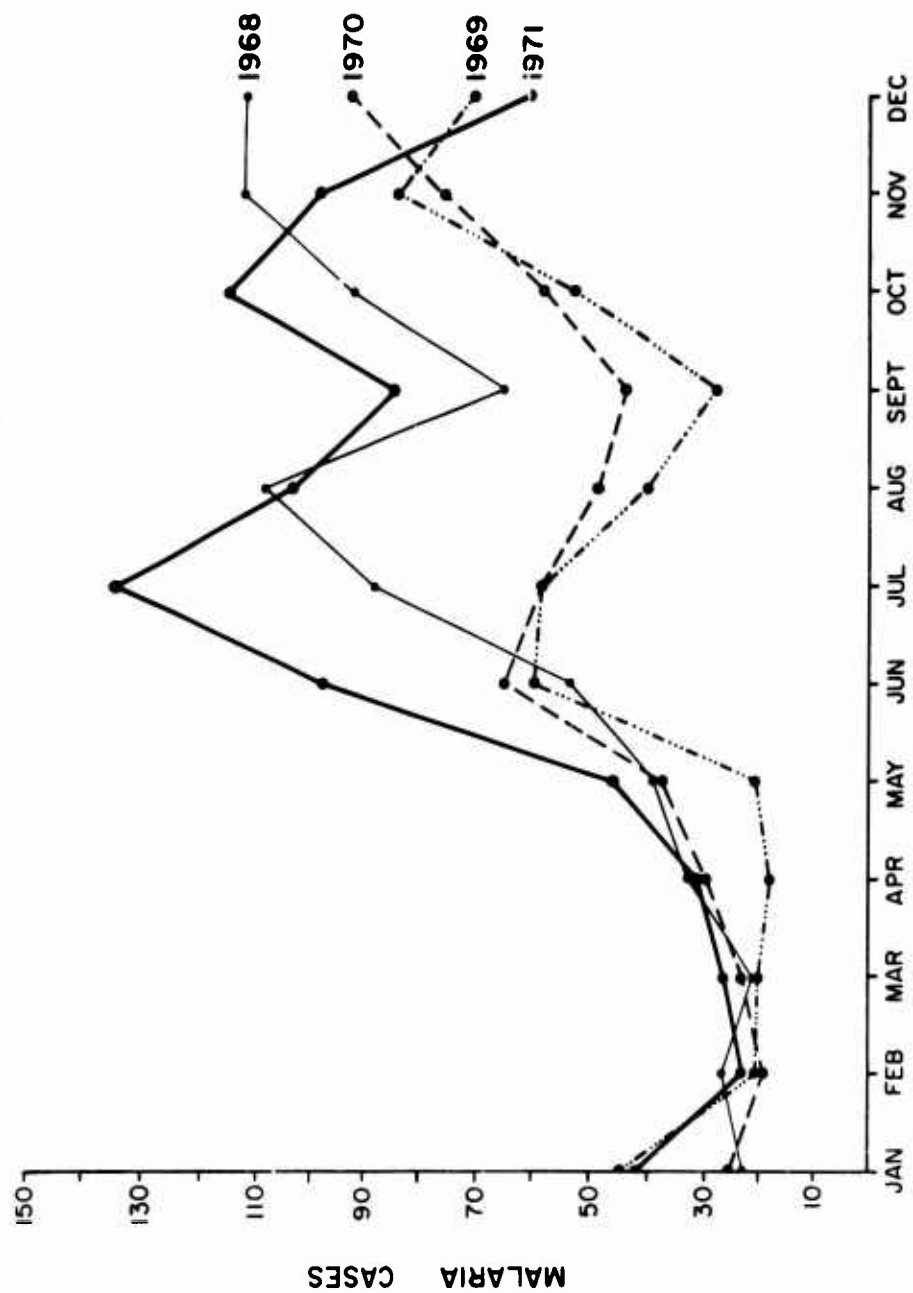
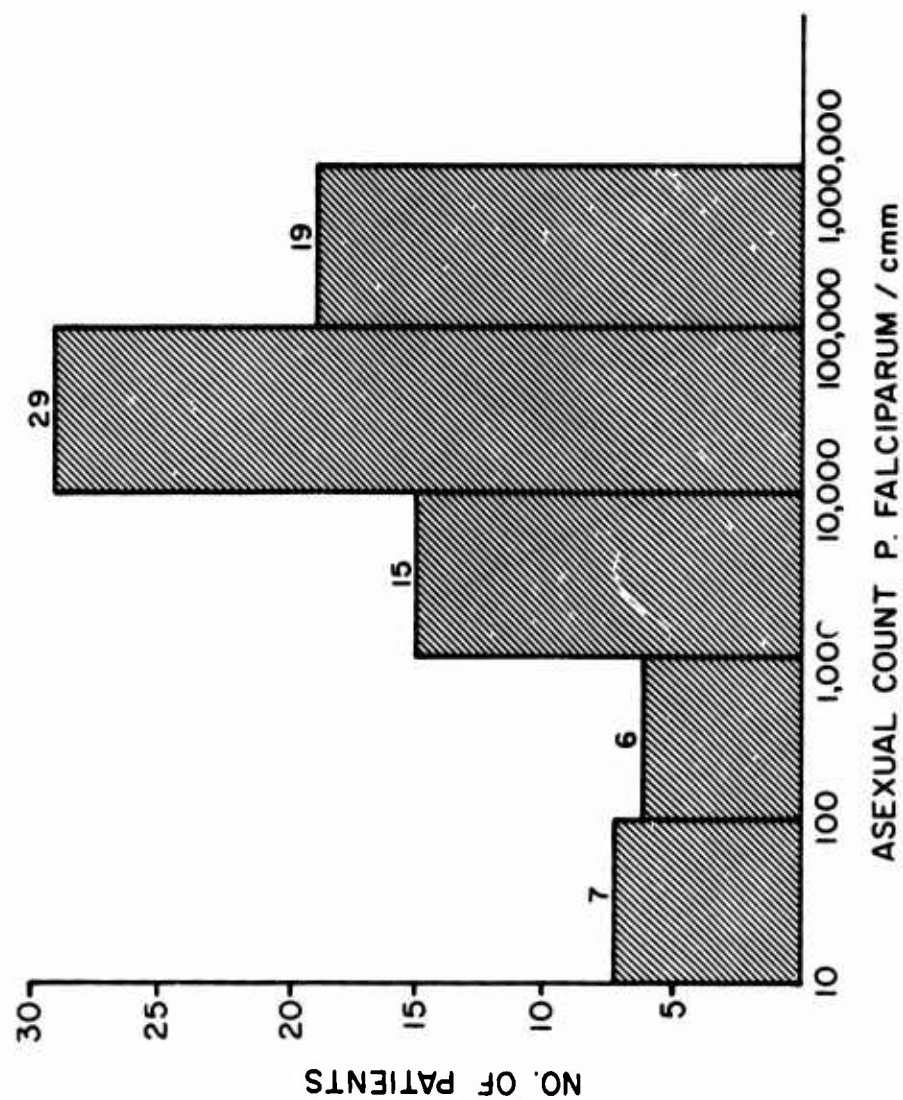


FIG. 3
PRACHINBURI HOSPITAL
DISTRIBUTION OF PARASITE COUNTS



and November. There was little malaria between January and June. Further details for 1971 are shown in Table 1a. and b.

Between 20 November and 19 December 1972 SMRL diagnosed 76 patients as having falciparum malaria and 8 as having vivax malaria. Twelve other individuals diagnosed as having malaria did not pass through the SMRL Laboratory. Thus malaria was the working diagnosis in 96 people.

The distribution of the parasite counts of P. falciparum inpatients are shown in Figure 3. The largest group had counts between 10,000 and 100,000/cmm (same as at Trad). There was a tendency for women to have lower parasite counts (Table 2). Ninety-six individuals were given antimalarial therapy and the frequency of each type of medication is shown in Table 3. (Patients in the SMRL therapeutic study are not included). The SMRL team screened most of the outpatients suspected of having malaria and the results are shown in Table 4.

It is concluded that:

1. Malaria is much less prevalent at the Prachinburi Hospital (fewer than 900 cases annually) than at the Trad Hospital (about 3000 cases annually).

2. Intravenous quinine is the most popular form of therapy and Fansidar is next.

2. Evaluation of WR 33063 in the Treatment of Acute Falciparum Malaria

The therapeutic efficacy of an investigational phenanthrenemethanol antimalarial, WR 33063, has been studied. This study was carried out at the Prachinburi Provincial Hospital, in Northeast Thailand, during a four-month period in mid-1972. Male patients presenting with acute, uncomplicated falciparum malaria with between 1,000 and 100,000 asexual parasites per cubic millimeter of blood were selected for study and hospitalized six days. Subjects were randomly assigned to either the "Study Group" or the "Control Group". The "Study Group" received WR 33063, 600 milligrams every eight hours for six days. The control group was treated with quinine sulfate, 625 milligrams every eight hours for

six days. Twice daily during hospitalization each subject was examined and capillary blood taken for quantitative parasite counts. Subjects were followed after discharge and capillary blood was collected for quantitative parasite counts on days 14, 21 and 28.

Fifty-one patients met the criteria for admission and were treated, 25 with WR 33063 (Study Group) and 26 with quinine sulfate (Control Group). The mean ages for the Study and Control Group were 28.1 and 25.5 years and the mean admission asexual parasite counts 29,492 and 25,272 parasites per cubic millimeter of blood respectively. All patients admitted to the study completed the specified period of hospitalization. The mean asexual parasite clearance time for the Study Group was 66.4 hours, compared with 65.1 hours for the Control Group ($t=0.21$, $0.90 > p > 0.80$). The mean fever defervescence times were 58.5 and 59.7 hours for the Study and Control Groups, respectively ($t=0.12$, $0.95 > p > 0.90$).

Symptoms of fever, insomnia, nausea and vomiting, abdominal pain, constipation, and myalgia were expressed slightly more often by Study Group subjects. Control Group subjects complained of headache slightly more often, and of tinnitus, blurred vision, chest pain, and diarrhea much more commonly. Complaints of dizziness and anorexia were equally distributed in both groups. Complaints voiced during the day of admission were not included (Table 1). Several subjects had biochemical evidence of hepatic dysfunction on admission. In no subject in the Study or Control Groups did serum bilirubin, alkaline phosphatase, creatinine, or transaminase values either become abnormal during therapy or rise to levels greatly in excess of admission values (Table 2).

Twenty-two of 25 Study Group subjects (88%) completed the followup period. The three who did not were re-treated for vivax malaria on day 21 (two subjects) and day 23 (one subject). All had been falciparum negative by smear to that time. Of the 22 subjects completing follow-up, 20 (90%) remained smear negative through day 28 and were considered cured. Of the two patients not cured, one infection was unresponsive (R3 pattern) and was successfully treated with intravenous followed by oral quinine. The other subject was not parasitemic until the day 28 follow-up (R1) and was admitted to a later SMRL study.

TABLE 1
REPORTED SYMPTOMS, WR 33063 AND
QUININE SULFATE-TREATED PATIENTS

Symptom	WR 33063 (25 Patients)	Quinine Sulfate (26 Patients)
Headache	8	11
Fever	6	5
Dizziness	2	2
Tinnitus	4	12
Insomnia	4	2
Blurred Vision	0	3
Chest Pain	0	2
Anorexia	4	4
Nausea, Vomiting	3	2
Abdominal Pain	4	2
Diarrhea	1	4
Constipation	1	0
Myalgia	2	0

TABLE 2
BIOCHEMICAL STUDIES, WR 33063 - AND
QUININE SULFATE - TREATED PATIENTS

Serum Specimens	MEAN (RANGE) SERUM VALUES				
	Bilirubin ¹				
	Direct	Total	Alkaline Phosphatase ²	Creatinine ³	SGOT ⁴
WR33063 day 0	0.56(0.1-3.0)	1.29(0.3-3.8)	2.53(0.8-6.1)	1.30(0.6-6.5)	39.1(15-77)
day 3	0.44(0.0-1.2)	0.78(0.1-1.7)	2.46(1.1-5.1)	1.18(0.6-8.5)	30.2(11-72)
day 6	0.32(0.0-0.9)	0.78(0.1-1.2)	2.38(1.1-4.7)	0.96(0.5-5.5)	31.9(11-106)
day 28	0.25(0.0-0.9)	0.57(0.1-1.5)	2.87(1.3-7.0)	0.79(0.5-1.3)	26.1(13-59)
QUININE day 0	0.69(0.1-5.1)	1.34(0.3-6.1)	2.07(1.0-4.7)	1.39(0.5-6.8)	29.1(14-75)
day 3	0.64(0.1-4.2)	0.91(0.2-4.9)	1.90(0.8-4.2)	1.22(0.5-5.3)	24.9(3-38)
day 6	0.46(0.0-3.4)	0.68(0.1-3.7)	1.86(0.9-3.9)	1.04(0.6-3.2)	23.8(14-55)
day 28	0.23(0.0-0.6)	0.50(0.1-1.1)	2.53(1.4-5.7)	0.78(0.5-1.2)	25.9(17-41)

- 1 Milligrams percent
- 2 Sigma units
- 3 Milligrams percent
- 4 Sigma-Frankel units

Twenty-one of the 26 Control Group subjects (81%) completed follow-up. One of those who did not was re-treated for vivax malaria (day 20); four others changed their residence and were lost at day 17 (one subject), at day 21 (one subject), and at day 28 (two subjects). All five had remained falciparum negative until their loss to the study. Of subjects completing follow-up, 20(95%) were cured. The single infection not cured responded partially (R2 pattern); this patient was re-treated with chloroquine-primaquine but did not return for follow-up.

The circumstances of follow-up do not exclude the possibility of reinfection. It is possible that the falciparum parasitemia found in one Study Group subject on day 28 represents a new infection. A more likely explanation for the vivax parasitemias found is that they represent relapsing infections suppressed by drug treatment.

The investigational drug employed in this study appears comparable in its therapeutic efficacy to quinine sulfate and somewhat better tolerated by the study subjects.

3. Comparison of Dapsone-Pyrimethamine and Sulfadoxine-Pyrimethamine Combination in the Treatment of Acute Falciparum Malaria.

Combinations of diaminodiphenylsulfone (dapsone) and pyrimethamine have been shown effective in the therapy of chloroquine-sensitive falciparum infections. In the one reported, preliminary study of the treatment of presumably chloroquine-resistant infections, results were disappointing. Because information on dapsone-pyrimethamine therapy of chloroquine-resistant infections is scanty and because of its potential therapeutic usefulness, the Ministry of Public Health expressed interest in a controlled evaluation.

The objective was to study the therapeutic efficacy of a combination of dapsone, 200 milligrams, with pyrimethamine, 25 milligrams (D-P), in comparison with that of sulfadoxine, 1000 milligrams with pyrimethamine 50 milligrams (S-P), both given as a single dose. The latter regimen is the standard single-dose antimalarial treatment administered at Prachinburi Hospital, the study site.

This study was carried out over a five-month period, at the Provincial Hospital, Prachinburi Province, Northeast Thailand.

Patients (both male and female) presenting with acute uncomplicated falciparum malaria with between 1,000 and 100,000 asexual parasites per cubic millimeter of blood were selected for study and hospitalized for 6 days. Subjects were randomly assigned to either the dapson-pyrimethamine (D-P) or sulfadoxine-pyrimethamine (S-P) groups and treated. Twice daily during hospitalization, each subject was examined and capillary blood taken for quantitative parasite counts. Subjects were followed after discharge and capillary blood for quantitative parasite counts was collected on days 14, 21 and 28.

Forty-four patients met admission criteria and were admitted to the study. Twenty-two (16 males, 6 females) were treated with dapson-pyrimethamine (D-P) and a like number (18 males, 4 females) with sulfadoxine-pyrimethamine (S-P). The mean ages for the D-P Group were 25.2 years and 22.0 years and, for the S-P Group, 27.4 years and 28.3 years for males and females respectively. The mean admission asexual parasite counts (parasites per cubic millimeter of blood) for the D-P Group were 22,573 and 28,373 and, for the S-P Group, 29,834 and 22,290 for males and females respectively.

Forty-three of the 44 patients admitted to the study completed the specified period of hospitalization. The mean parasite clearance time for the D-P Group was 65 hours (range: 36-109) and for the S-P Group was 62 hours (range: 33-84). The mean fever defervescence time for the D-P Group was 64 hours (range: 14-112) and for the S-P Group was 56 hours (range: 18-106). Neither the mean parasite clearance times ($t=0.684$, $0.50 > p > 0.40$) nor mean fever defervescence times ($t=1.013$, $0.40 > p > 0.30$) of the D-P and S-P Groups were significantly different. There were no sex differences in either of these variables.

Both drug regimens were well tolerated, with similar symptomatic complaints reported by the study subjects (Table 1). Symptoms of tinnitus and gastrointestinal irritation were reported somewhat more often by patients receiving D-P than by those receiving S-P.

Follow-up was completed on 38 of the 44 subjects selected for study (86%). Four of 21 D-P Group subjects were cured (19%), compared with 14 of 17 S-P Group subjects (82%). This difference is statistically significant ($X^2=12.6$, $p<0.0001$). All 17 drug failures in the D-P Group were of the R1 type. In the S-P Group,

TABLE 1
REPORTED SYMPTOMS, DAPSONE-PYRIMETHAMINE AND
SULFADOXINE-PYRIMETHAMINE-TREATED PATIENTS.

Symptom	Dapsone-Pyrimethamine (22 Patients)	Sulfadoxine-Pyrimethamine (22 Patients)
Headache	14	15
Fever	9	10
Chills	0	2
Dizziness	4	2
Weakness	1	2
Tinnitus	4	0
Insomnia	3	1
Chest Pain	0	1
Anorexia	5	3
Nausea, Vomiting	4	2
Abdominal Pain	6	0
Diarrhea	0	1
Myalgia	0	1
Muscle Twitching	0	1

one R1 and two R2 failures were noted.

The dapsone-pyrimethamine combination employed in this study does not appear sufficiently efficacious to warrant its use in the treatment of acute falciparum malaria. Conversely, the efficacy of the standard sulfadoxine-pyrimethamine regimen in the treatment of acute falciparum malaria is confirmed.

4. Hospital Survey of Malaria in Trad, Southeast Thailand.

The objectives of these studies are:

1. To determine the clinical picture of malaria at Trad Hospital.
2. To perform parasite counts on patients suspected of malaria, as a service for the Trad Hospital.
3. To screen patients with falciparum malaria suitable for a SMRL study.

SMRL studies were resumed at the Trad Provincial Hospital on 11th January 1973. The SMRL malaria unit occupies an unused ward located above the Male Medical Ward, by courtesy of Dr. Sanong Kosakal the Medical Director of the Hospital. Half the area has been converted into a spacious laboratory and the remainder into living quarters for the technicians and drivers. The clinic is open 0600 to 2200 daily. Anybody may walk in and have a malaria smear done. Other patients are referred from the Hospital outpatient clinic.

Patients with a positive smear are interviewed (current symptoms, history of previous malaria), examined (fever and splenomegaly as a minimum) and a quantitative parasite count is made. In consultation with the Trad physicians, the patients are admitted to the hospital or treated as outpatients.

Up to 31 March 1973, the clinic had been open for 80 days and had examined 1101 patients, of whom 461 were infected with Plasmodium falciparum and 71 with P. vivax (Table 1). The largest group of patients with falciparum malaria had a parasite count between 10,000 and 100,000 per cmm. The distribution of

TABLE 1.

MALARIA DIAGNOSES IN THE SMRL OUTPATIENT CLINIC,
TRAD HOSPITAL 1973

	PATIENTS SCREENED	FALCIPARUM	VIVAX*
JANUARY (11th-31st)	167	74	11
FEBRUARY	317	109	19
MARCH	<u>617</u>	<u>278</u>	<u>41</u>
TOTAL	1,101	461	71

* P. vivax comprised 13% of all malaria cases.

TABLE 2.

SMRL MALARIA CLINIC AT TRAD. DISTRIBUTION OF PARASITE
COUNTS OF P. FALCIPARUM IN MALES AND FEMALES

Parasite Count/cmm	0-100	100-1000	1000-10000	10,000-100,000	100,000+	TOTAL
No. Males	35 (8%)	70 (16%)	131 (34%)	164 (38%)	34 (8%)	434
Average Age	31	29	25	27	24	28
No. Females	3 (10%)	9 (33%)	10 (36%)	5 (18%)	1 (3%)	28*
Average Age	42	26	18	25	-	24

* Many females with malaria were not seen in the clinic.

parasite counts was similar to that seen in the Prachinburi Hospital in Central Thailand. There was a tendency for parasitemia to be lower in women and in older people (Table 2).

Splenomegaly was present in 31% (54/172) of patients with falciparum malaria and in 45% (13/29) of patients with vivax malaria (not all patients were examined).

By doing parasite counts before admission and when requested afterwards, the SMRL clinic greatly assists in the management of the patients with malaria.

5. Gastrointestinal Absorptive Function, Quinine Absorption, and Parasite Response in Acute Falciparum Malaria.

Persons ill with acute falciparum malaria sometimes present with gastrointestinal symptoms, of which anorexia, nausea, and vomiting are the most common. Gastrointestinal absorptive function has been shown to be abnormal in patients with acute falciparum malaria. Olsson and Johnston found impaired d-xylose absorption and histopathologic changes in intestinal biopsy specimens taken from malarious American soldiers. These observations were extended by Karney and Tong using additional indicators of absorptive function. In neither of the above studies were levels of orally-administered antimalarial drugs and parasite responses measured or correlated with absorptive function.

We plan to measure concurrently and correlate gastrointestinal absorptive function, antimalarial drug levels, and parasite responses in patients ill with acute falciparum malaria, to determine whether malabsorption in malaria has any clinical significance. Two groups of patients will be included, a falciparum infected Study Group and an uninfected Control Group.

Study Group: Twenty male patients admitted to Trad Provincial Hospital and assigned to the Quinine Group of the WR 30090 protocol will be studied. The course of hospitalization and procedure sequence will be modified to include the d-xylose absorption studies and collection of specimens for serum quinine and serum carotene determinations. The d-xylose test will be performed on each Study Group subject twice during hospitalization, on days 1 and 6. A serum specimen for serum carotene determination will

also be collected on day 28. A single stool specimen collected during hospitalization will be studied for ova and parasites.

Control Group: Twenty male patients admitted to Trad Provincial Hospital with diagnoses other than malaria or those involving the gastrointestinal or urinary tracts will be studied. Demographic data on these subjects will be taken and they will be administered the d-xylose test once, on day 1 of hospitalization. The subject selected will be the next acceptable, willing admission following the admission of the "Study Group" patient. A single stool specimen collected during hospitalization will be studied for ova and parasites.

To date, sixteen patients ill with acute falciparum malaria treated with quinine sulfate (Study Group) and twelve patients with diagnoses other than malaria (Control Group) have been studied. When twenty patients in each group have been studied, the similarity of the Study and Control Groups will be tested by comparing the median ages, and by comparing other demographic variables of interest such as marital status and place of residence. Days 1 and 6 d-xylose and serum carotene test results in the Study Group will be compared and contrasted to those of the Control Group. The degree of relationship of d-xylose absorption, serum carotene levels, plasma quinine levels, and parasite responses will be measured by calculation of correlation coefficients.

6. Comparison of WR 30090, WR 33063 and Quinine for Falciparum Malaria in Southeast Thailand.

The objective of these studies is to compare the action against P. falciparum and the toxicity of WR 33063, WR30090 and Quinine in Southeast Thailand.

The studies were performed at the Trad Provincial Hospital. Chloroquine resistant falciparum malaria is highly prevalent in Trad (Colwell, S.E. Asia J. Trop. Med. Pub. Hlth. 3:190, 1972). Male patients with falciparum parasitemia of between 1,000 and 100,000 per cmm gave their informed consent. Quantitative parasite counts were performed in the hospital from day 0 to day 6 and at follow-up on days 14, 21 and 28. Blood counts and serum biochemistries were done on day 0, 3, 6 and 28. The patients were randomly assigned to one of three treatment regimens. The following doses were given orally every 8 hours for 6 days: WR33063,

TABLE 1.
INITIAL RESPONSE OF FEVER AND PARASITEMIA TO THERAPY

Drug	No. Patients	Mean Parasite Count (/cmm)	Mean Parasite Clearance (hours)	Mean Highest Temperature	Mean Fever Clearance (Hours)
WR33063	36	26,000	74	102.4	52
WR30090	39	21,000	66	102.1	58
QUININE	38	25,000	66	101.8	64

TABLE 2
RADICAL CURE RATES AND INCIDENCE OF P. VIVAX DURING FOLLOW-UP

	Antimalarial Effect				Follow-up not Complete	Lost to Follow-up	% Cure Rate	P. vivax During Follow-up
	S	RI	RII	RIII				
WR33063	19	2	0	1	11	3	86%	30%
WR30090	16	1	0	0	17	5	94%	30%
QUININE	17	2	0	0	15	4	89%	40%

TABLE 3

NUMBER OF PATIENTS ON EACH DRUG WHO
DEVELOPED THE SYMPTOMS LISTED*

	WR33063 36 Patients	WR30090 39 Patients	QUININE 38 Patients
ANOREXIA	2	1	4
BACKACHE	8	8	4
BLURRED VISION	0	0	2
HEADACHE	15	23	18
MEDICATION INTERRUPTED	0	1	5
POSTURAL HYPOTENSION	0	0	4
TINNITUS	0	2	16
WEAKNESS	8	2	7
TOTAL	33	37	60

* Only those symptoms are listed which showed a difference between the groups.

600 mg; WR 30090, 250 mg; and Quinine Sulfate, 540 mg.

WR33063 is 3-bromo-10 (a-hydroxy-B-(N, N-diheptylamino) ethyl) phenanthrene hydrochloride, and WR30090 is 6, 8-dichloro-a(dibutyl-aminomethyl)-2-(3'-4'-dichlorophenyl)-4 quinoline-methanol hydrochloride.

The project was begun on 11 January 1973. By 18 April 114 patients had been studied. In hospital all 3 drugs were effective in controlling the disease (Table 1). WR33063 was the most effective with respect to the control of fever but was slower in clearing parasitemia. The cure rates with each drug were about 90% (Table 2). Unfortunately about 40% of the patients developed P. vivax parasitemia during follow-up. Toxicity was greater with Quinine (Table 3).

In general the patients were fit for discharge on day 3, so that the extra 3 days in the hospital for the six day course of therapy was an inconvenience. WR30090, requiring only 1 capsule per dose, is more acceptable than WR33063 which necessitates the swallowing of 3 capsules.

The results of these studies to date may be summarized as follows:

1. WR33063 has so far proved effective and non-toxic.
2. WR30090 also proved effective and non-toxic.
3. Quinine was effective but more toxic.
4. All 3 drugs were inconvenient in having to be administered for 6 days. The ideal antimalarial should take 3 days or less for administration of the full dosage.

7. Intravenous Quinine for Falciparum Malaria.

The objective of these studies was to determine the optimum dose of quinine when intravenously infused for severe falciparum malaria.

In a recent study, quinine by intravenous infusion proved more effective than oral quinine in the radical cure of recrudescant falciparum malaria.¹ Intravenous quinine is often used in the management of severe acute falciparum malaria but many physicians are deterred by the well-known toxicity of the drug. Chloroquine

by intravenous infusion is often preferred therapy. Therefore it is important to evaluate the role of intravenous quinine in this situation. The study was begun at Trad on 17 January 1973. Patients with falciparum malaria are considered for study. Criteria for selection are a parasite count over 100,000 per cmm or clinically severe illness. 24 patients have so far been studied in detail and the response of other patients also noted. Quantitative parasite counts are performed twice daily.

Initially most adult patients received 20 grains quinine in the first liter of intravenous fluid. Toxicity (e.g. tinnitus) occurred in some patients on this dosage but was absent when the initial liter contained only 10 grains quinine. This is now the standard dose for patients not in coma, whereas patients with evidence of cerebral malaria initially receive 20 grains quinine in the first liter. Clinical and parasitemic response to the lower dose is usually excellent and further intravenous therapy is usually unnecessary. The first liter is infused in anything from 30 minutes to 6 hours depending on the clinical picture. Oral therapy is then given, which is usually quinine with or without tetracycline. (At Prachin-buri Hospital Fansidar is often the drug combination used following the intravenous quinine).

Likewise in infants and small children, intravenous quinine has proved very successful, in fact lifesaving. Infants usually receive about 2.5 grains in 250 ml fluid and small children about 5 grains in 500 ml.

In comatose patients shots of 4 mg Decadron (dexamethasone) are injected into the drip tubing about every 6 hours.

Since intravenous quinine has proved so effective in reducing both symptoms and parasitemia, this mode of therapy is now being increasingly used in patients who are moderately but not seriously ill. Intravenous quinine appears less toxic than oral quinine.

The majority of patients with severe falciparum malaria respond rapidly to an intravenous infusion of 1 liter of saline to which is added 10 grains quinine dihydrochloride. The average time of infusion is 3 hours. Further doses more slowly infused are occasionally required. Normal saline is the preferred solution because hyponatremia has been detected in 75% of patients with malaria in

Thailand.²

8. Comparison of Amodiaquine and Chloroquine for Falciparum Malaria in Thailand.

Studies are in progress to compare the antimalarial efficacy and toxicity of amodiaquine and chloroquine. Both these antimalarial drugs are 4-aminoquinolines and Rieckmann (J. Am. Med. Assoc. 217:573, 1971) has claimed that a strain of P. falciparum from Vietnam was more susceptible to amodiaquine than to chloroquine.

The studies were conducted at Trad Provincial Hospital. Colwell and associates, using an in vitro test, have found that chloroquine resistant strains are highly endemic in Trad. The medical community in Trad frequently uses chloroquine by the oral or parenteral route for the treatment of falciparum malaria. Therefore it seemed appropriate to evaluate these 2 agents under controlled hospital conditions.

The study was begun on 13 March 1973. Patients with mild to moderate falciparum malaria were selected for study. Quantitative parasite counts were performed before therapy and twice daily during the hospital course. A hematocrit and WBC were determined on days 0, 3 and 6. Urines for drug level determinations were obtained before therapy and daily during drug administration.

Antimalarials were administered by a study physician. The patient was observed swallowing the drug and then water afterwards. The dosage of amodiaquine and chloroquine were the same. On day 0 the initial dose was 600 mg base followed by 300 mg 6 hours later. Both on day 1 and day 2, a 300 mg dose was administered in the morning. The total dose for both drugs was 1,500 mg in accordance with W.H.O. criteria. Also both preparations were not enteric coated. The "Nivaquine" brand of chloroquine and the "Camoquin" brand of amodiaquine were used.

Twelve patients have so far been admitted to the study. 5 have received chloroquine and 7 amodiaquine. The results are shown in Table 1. Chloroquine has proved slightly more effective than had been expected. Complete clearance of parasitemia occurred in 2 patients but in the other 3 the effect was only partial

The term S or RI refers to the situation where parasitemia has been cleared in hospital but follow-up has not been achieved. Thus the final result in these patients would have been a radical cure or a recrudescence. Complete clearance of parasitemia was achieved in 5 patients treated with amodiaquine. The other 2 patients, treated with amodiaquine, left the hospital before the parasitemia had completely cleared. Of special interest was a patient who did not respond to chloroquine (RII response) and had to be treated with intravenous quinine. Twenty-one days after discharge he developed a recurrent attack (probably a recrudescence). There was a satisfactory initial response (S or RI) when this attack was treated with amodiaquine.

Our results to date indicate that:

1. Falciparum malaria in Southeast Thailand continues to be highly resistant to chloroquine. This confirms previous work.
2. Amodiaquine may prove to be more effective than chloroquine.

TABLE 1.

FALCIPARUM MALARIA IN THAILAND

RESPONSE TO AMODIAQUINE OR CHLOROQUINE

	S or RI*	RII	RIII	Unknown
CHLOROQUINE (1.5g)	2	2	1	-
AMODIAQUINE (1.5g)	5	0	0	2

* Initial complete clearance of parasitemia occurred but follow-up was not achieved.

9. Pyrimethamine Resistant Falciparum Malaria in Thailand.

The pyrimethamine response of naturally acquired falciparum malaria in Thailand has not been studied. Because it is a widely used antimalarial drug either alone or in combination, its

therapeutic efficacy was tested.

The studies were conducted at the Trad Provincial Hospital in February 1973. The patients were first seen at the SMRL Malaria Clinic and quantitative parasite counts performed. Mild cases of falciparum malaria were selected for study. Informed consent was obtained in all patients.

Three patients have been treated:

1. The first patient was seen on 6 February 1973. He was a 40 year old farmer with fever, headache and weakness for a few days. The only therapy was 2 unidentified tablets 2 days previously. He said that he had had an attack of malaria 1 month earlier. His temperature was 98.6°F on admission. Splenomegaly was present throughout the 6 days in the hospital. His asexual count of P. falciparum was 810/cmm (Table 1). 50 mg pyrimethamine was given daily for 3 days (total dose 150 mg). The parasitemia decreased but rose again on the 6th day at which time quinine was administered on an out-patient basis for 6 days. Parasitemia was absent at follow-up examinations on days 14, 21 and 28.

2. This 32 year old man gave a history of fever and headache for one month. He had taken no antimalarials for 6 days. His asexual count was 8580/cmm and there was a partial response to the standard 150 mg course of pyrimethamine (Table 2); however, on day 4 his count rose sharply to 1377/cmm and quinine was administered. The patient did not attend follow-up.

3. This 19 year old man gave a 3 day history of fever, weakness and myalgia. His parasite count of 950/cmm was decreased by pyrimethamine therapy but a resurgence of parasitemia on day 6 responded to quinine therapy (Table 3).

In all 3 patients, the falciparum parasitemia made only a temporary response to pyrimethamine. This is an RII response under W.H.O. criteria. Pyrimethamine resistance has been previously shown in artificially induced infections in the USA using a strain isolated from a white man in Thailand (See references 3, 4, 5).

This report confirms that pyrimethamine resistance is found in natural infections in Thailand.

TABLE 1.

THE PARASITEMIA OF P. FALCIPARUM IN CASE 1
 ONLY PARTIALLY RESPONDED TO PYRIMETHAMINE(RII RESPONSE)

DATE	DAY	TIME	ASEXUAL COUNT	GAMS	THERAPY	COMMENT
6 FEB 1973	0	1000 1400	810 3812	72	Pyrimethamine 50 mg	RII Response
	1	0800 1400	200 20	Rare 20	Pyrimethamine 50 mg	
	2	0800 1400	40 100	20 20	Pyrimethamine 50 mg	
	3	0800 1400	Rare Rare	20 20		
	4	0800 1400	Rare Rare	20 20		
	5	0800 1400	Rare Rare	20 20		
	6	0800	40	20	Quinine(6 Days)	
	14		0	45		
	21		0	Rare		
	28		0	0		
					S Response	

TABLE 2.

CASE 2. PYRIMETHAMINE THERAPY OF FALCIPARUM MALARIA
FOLLOWED BY RII RESPONSE

DATE	DAY	ASEXUAL COUNT	GAMS	THERAPY	COMMENT
7 FEB 1973	0	8580 7007	0 0	Pyrimethamine 50 mg	RII Response
	1	3160 1720	0 0	Pyrimethamine 50 mg	
	2	1140 660	0 0	Pyrimethamine 50 mg	
	3	960 580	0 0		
	4	80 1377	0 81		
	5	3185		Quinine (6 Days)	

TABLE 3.

CASE 3. RII RESPONSE OF FALCIPARUM MALARIA TO PYRIMETHAMINE

DATE	DAY	ASEXUAL COUNT /cmm	GAMS	THERAPY	COMMENT
19 FEB 1973	0	940	40	Pyrimethamine 50 mg	RII Response
	1	1296 20	162 20	Pyrimethamine 50 mg	
	2	20 Rare	20 20	Pyrimethamine 50 mg	
	3	Rare Rare	72 48		
	4	Rare 192	20 24		
	5	40 40	20 40		
	6	1512 1080	0 40	Quinine (6 Days)	
	7	360	20		
	14	0	0		

10. The Suppression of Plasmodium Falciparum and Plasmodium Vivax Parasitemias by a Dapsone-Pyrimethamine Combination.

Combinations of sulfones with pyrimethamine have been shown not to be effective in the treatment of persons clinically ill with chloroquine-resistant falciparum parasitemias. There is little information about the performance of these combinations in the long-term suppression of such parasitemias. Therefore we studied the effectiveness of a combination of dapsone 100 milligrams and pyrimethamine 12.5 milligrams in suppressing parasitemias in an area endemic for chloroquine-resistant P. falciparum and for P. vivax malaria.

This study was carried out over a seven-month period in two villages in Prachinburi Province, Northeast Thailand. Four hundred and fifty randomly selected villagers 10 years of age or older were assigned to one of three medication groups. These groups were comparable in number, median age, proportion of males (Table 1), and later, in number and type of study dropouts (Table 2). Subjects received weekly, under a double-blind design, either dapsone, 100 milligrams, and pyrimethamine, 12.5 milligrams, or dapsone-pyrimethamine plus chloroquine, 300 milligrams of base, or chloroquine alone. Each subject was visited weekly, at which time medication was administered, capillary blood for thick-thin malaria smears obtained, and a history of illness since the previous visit taken.

Four hundred and ten subjects (91%) completed the 250week course of medication. Preliminary data compilation (Table 3) suggests that falciparum attack rates of the groups receiving dapsone-pyrimethamine, with or without chloroquine, were three-to four fold less than the attack rate of the group receiving chloroquine alone. The number of weekly positive smears in the former groups was six-to eight-fold lower than in the group receiving chloroquine alone. When slide reading is complete, weekly infection rates will be studied to determine patterns of breakthrough. Further data analysis will also reveal whether the parasite densities and/or illness experienced by the three study groups were different. Vivax infection rates were 0.6% in Group 1, 2.8% in Group 2, and 0.7% in Group 3.

TABLE 1
INITIAL COMPARABILITY OF STUDY GROUPS

Group	Number Subjects	Median Age (Years)	Proportion Males
1(dapsone-pyrimethamine, chloroquine)	156	24.0	0.48
2(dapsone-pyrimethamine)	152	27.0	0.56
3(chloroquine)	142	28.0	0.49

TABLE 2
COMPARABILITY OF STUDY DROPOUTS

Group	Number Beginning	Number Dropouts	Number Completing	Reason for Dropout
1	156	14	142	Moved 11, Refused 3
2	152	14	138	Moved 11, Refused 3
3	142	12	130	Moved 11, Refused 1

TABLE 3

FALCIPARUM ATTACK RATES AND NUMBER OF PARASITEMIC
EPISODES IN SUBJECTS COMPLETING STUDY, BY GROUP

Group	Number Subjects	Number(%) Infected	Number Episodes * Parasitemia (Avg.)
1	142	25(17.6)	68(2.7)
2	138	20(14.5)	49(2.5)
3	130	71(54.6)	393(5.7)

* Based on 26 blood films from each subject

We conclude that weekly dapsone-pyrimethamine, with or without chloroquine, appears to effectively suppress falciparum infections in an area endemic for chloroquine-resistant strains of the parasite.

11. Ecology of Malaria Vectors

The objective of these studies is to investigate the bionomics and population dynamics of known and potential vectors of human malaria in Southeast Asia and their relationship to the dissemination of chloroquine resistant strains of P. falciparum.

Specific factors being studied in the process of defining actual and potential vector species in Thailand include the following: incidence of malarial oocysts and sporozoites in wild anopheline populations, susceptibility of colonized strains of Anopheles to infection with P. falciparum, patterns of biting activity of vector species, ovipositional habits of anopheline mosquitoes and the viability of their eggs under various environmental conditions.

Malaria field studies in Prachinburi province. Entomological field studies were continued in Prachinburi Province in support of a longitudinal study of the epidemiology of malaria in the area. Results of studies described in the previous Annual Report⁶ indicated that the risk of malaria was greater in the forest than in the village setting. Anopheles balabacensis was shown to be the predominant malaria vector in the area. Between April and November, 1972 anopheline collections from human-bait and vegetation were made simultaneously on nine nights in forest and village locations to compare vector densities in the respective areas. Except for one night, these collections were made between dusk and dawn. In these collections, a total of 403 A. balabacensis were collected, biting humans and resting on vegetation in vicinity of collectors in the forest, as opposed to only 30 in the village site. The number collected biting per man per night is shown in Table 1 for both locations. In addition, 421 A. balabacensis were collected biting or resting in the forest on seven separate nights, while in the village only 55 A. balabacensis were collected on ten nights. The breakdown of the number of these mosquitoes collected biting per man per night is shown in Table 2. The only A. balabacensis found infected with either oocysts or sporozoites were collected in the forest during the dry season in January and February 1973. In January four of 92 collected biting contained either oocysts and/or

sporozoites while one additional A. balabacensis collected resting also was found to contain oocysts. Two of 47 A. balabacensis collected biting on one night in February contained oocysts. Blood films were obtained from five people living in a nearby hut and trophozoites of P. falciparum were demonstrated in the blood from four of these individuals. One slide had falciparum gametocytes as well as asexual stages.

Efforts were made during this period to determine how A. balabacensis populations survive the extended dry season. Previous studies had shown that eggs were not important in over-dry season survival, and larvae were not found at the time either in Ban Bu Phram valley or at the edge of the forest. During the dry season covered by this report, A. balabacensis larvae were found in rock pools alongside partially dry stream beds on the steep slopes of surrounding hills and in shallow pits dug at stream margins by villagers as a source of water in the forest. Identical pools were dug out by SMRL personnel and A. balabacensis larvae were collected from these new pools within 10 days. Thus, it appears that during the dry season the life cycle of A. balabacensis is maintained in the forest and that breeding extends into the central portion of the valley during the rainy season as breeding sites become available there.

Susceptibility of A. balabacensis and A. minimus to infection with P. falciparum prior to and after standard chloroquine therapy. The suggestion has been made that the wide-spread use of chloroquine may have contributed to the rise of chloroquine resistant strains of P. falciparum.⁷ Romkaran & Peters⁸ have shown that the infectivity of a chloroquine-resistant strain of P. berghei for A. stephensi is enhanced after treatment with chloroquine. No differences were observed in the infectivity of drug sensitive strains after treatment with chloroquine. A study was undertaken to determine if chloroquine enhanced the infectivity of P. falciparum for A. balabacensis and A. minimus. Laboratory reared strains of these two species were fed simultaneously on 29 human subjects with P. falciparum infections in which gametocyte densities were at least 25 per cmm. Mosquitoes were fed on these subjects immediately prior to administration of 1500 mg chloroquine base (day 0) and on days 2 and 7 when treatment was completed. All patients were admitted to the district hospital in Phra Phutthabat for the first 7 days of study. During this period blood films were

Table 1. A. balabacensis collected biting man or simultaneous nights in the forest and village settings.

Month	No. nights	Forest		Village	
		No. Coll.	No./man night	No. Coll.	No./man night
Apr.	1	100	16.6	3	0.6
May	4	60	5.0	7	0.3
Jun	2	30	3.0	1	0.1
Aug*	1	4	2.0	1	0.5
Oct	1	7	3.5	2	0.5
Nov	1	8	4.0	5	1.3
Totals	10	209	6.15	19	0.48

*Collection between 1800-2400 hrs.

Table 2. A. balabacensis collected biting man on different nights in the forest and village settings

Month	No. nights		Forest		Village	
	Forest	Village	No. Coll.	No./man night	No. Coll.	No./man night
Apr.	-	2	-	-	4	0.4
Jun	3	5	90	5.6	18	0.9
Jul	-	2	-	-	6	1.4
Aug*	1	1	26	6.5	15	3.8
Jan*	1	-	92	30.6	-	-
Feb*	2	1	81	5.8	0	0
Totals	7	10	289	7.8	43	1.2

*Collections between 1800-2400 hrs.

fed on these subjects immediately prior to administration of 1500 mg chloroquine base (day 0) and on days 2 and 7 when treatment was completed. All patients were admitted to the district hospital in Phra Phutthabat for the first 7 days of study. During this period blood films were taken daily and examined for parasites. After the subjects were discharged from the hospital, follow-up blood films were taken on days 14, 21 and 28 to determine the status of chloroquine resistance, using WHO criteria. Mosquitoes which fed on these subjects were dissected 8 to 10 days later and the oocysts in their guts counted. The proportions of mosquitoes with oocysts (per cent positive) and the mean number of oocysts per infected mosquito (oocyst index) were used to compare the susceptibility of the two mosquito species to untreated and treated falciparum malaria.

Seventeen of the subjects were infectious to at least one mosquito species on either the pre- or one of the post-treatment feedings. Two of the subjects were not infectious on the pre-treatment feed but one of these was infective on the second day and the other was infective on day 7. A twenty-eight day follow-up was completed on 13 of 17 subjects. Ten of these exhibited an RI response and 3 were RII. Three subjects could not be followed after discharge and an additional subject was switched to quinine and tetracycline therapy prior to discharge. The results of the dissection of the groups of mosquitoes which fed on these subjects are shown in Table 3. There was a decrease in the median percent infected for both mosquito species after chloroquine treatment. These results suggest that chloroquine treatment did not enhance the infectivity of chloroquine resistant strains of P. falciparum; however, no RIII level resistant strains were studied.

In this study, there was little difference in the susceptibility of the two mosquito species to infection with falciparum gametocytes, although previous studies indicated that A. balabacensis were more susceptible than A. minimus. Techniques used were the same in both studies; however, in the present investigation higher proportions of A. minimus fed and more were dissected than previously. In addition, the larval diet in the A. minimus colony was changed between studies. This change resulted in greater longevity in the A. minimus colony.

Irritability of *A. balabacensis* to DDT. Tests to determine the irritability of *A. balabacensis* to DDT have been initiated, using the procedures recommended by the WHO. Initial results are inconclusive and further studies are planned.

Table 3. Results of dissections of *A. balabacensis* and *A. minimus* which were infected when fed simultaneously on 17 subjects either prior to or on day 2 or 7 after starting chloroquine treatment.

Species	Day	Percent Positive		Oocyst Index	
		Median	Range	Median	Range
<i>A. balabacensis</i>	0*	48	0-100	4.1	0-100
	2**	40	0-78	8.0	0-74
	7***	23	0-74	6.7	0-82
<i>A. minimus</i>	0*	49	1.0-120.2	2.6	1.0-25.0
	2**	38	1.0-29.2	2.2	1.0-9.5
	7***	26	2.1-46.7	3.5	2.5-32.9

* Day 0 - 29 subjects

** Day 2 - 23 subjects

***Day 7 - 19 subjects

12. Mosquito Fauna of Thailand

Work is in progress to collect, identify, catalogue and redescribe the mosquito species of Thailand. Information is also gathered on the distribution, larval habitats and other aspects of the bionomics of various species. The eventual goal is the production of monographs on the mosquitoes of the area, together with keys, handbooks and other identification aids, for use of workers in public health and associated fields.

Mosquitoes are collected from many areas of Thailand in connection with various studies on malaria and other arthropod borne diseases. Additional collections of a specialized nature are made to obtain a correlated series of larvae, pupae and adults for illustration and taxonomic studies. The majority of this material is shipped to the Smithsonian Institution for study by specialists in the Southeast Asia Mosquito Project (SEAMP).

During this year 173 mosquito collections were made in 3 provinces of Thailand. The majority of the collections were made in Chiangmai province. These collections resulted in 1035 pinned adults, 1490 slide mounts of larvae, larval and pupal skins and 50 slide mounts of terminalia. An additional 410 collections of mosquito larvae were received for identification, 560 larvae with parasites were mounted for further study and 1172 slides of Culicoides from light trap collections were made.

- 1). Anopheles: During this period adult female An. (An.) campestris were collected in an effort to establish a laboratory colony for experimental studies. This species is the most important species in the barbirostris group and is a vector of human malaria in western Malaysia. Adults of An. campestris resemble those of An. barbirostris, and identifications based on adult characters alone should be confirmed by examination of the immature stages, particularly the pupae. Larvae of this species were found in ground water habitats of various types including marshes, wells, hoof prints, ditches and swamps. Larvae of An. (An.) hodgkini, an uncommon species in barbirostris group, were collected from stream and rock pools in association with barbumbrosus, bengalensis and balabacensis larvae at the malaria study site in Prachinburi province.
 - 2). Culex: Studies of Subgenus Culex were continued. Species of Culex vishnui subgroup were collected in an effort to obtain female Culex pseudovishnui for sibling rearings.
 - 3). Aedes: Bamboo cup collections were made in an attempt to obtain rarer species of the Subgenus Stegomyia. A colony of Aedes craggi was established with material collected in Chiangmai province. Collections of Aedes albopictus, one of the commonest species in Thailand, were checked in an effort to find specimens of Aedes patricia and A. novalbopictus, species which closely resemble A. albopictus. All species in this subgenus can be distinguished by characters of the male terminalia.
13. Plasmodium Falciparum Infection Rates in Normal and Enzyme-Deficient Erythrocytes of Glucose-6-Phosphate Dehydrogenase Deficient Heterozygous Thai Women.

P. falciparum infection rates in normal and enzyme-deficient

erythrocytes of Thai women, heterozygous for glucose-6-phosphate dehydrogenase (G-6-PD) deficiency are being determined and compared.

Women, heterozygous for G-6-PD deficiency (an X-chromosome-linked trait), are mosaics: approximately half of their red blood cells are normal, the other half are G-6-PD deficient. The two cell populations can be distinguished histochemically by the methemoglobin elution method⁹. This technique is being applied to blood from Thai women who have malaria and are heterozygous for G-6-PD deficiency. Infection rates in both normal and enzyme-deficient red blood cells are being determined and compared. Hematocrit, reticulocyte count, red blood cell morphology, hemoglobin type, and G-6-PD activity (spectrophotometric assay) are also being determined.

To date, approximately 600 women and girls with malaria, who presented at the Provincial Hospital or Malaria Eradication Center in Chantaburi, have been tested by the methemoglobin elution technique. Approximately ten per cent have been classified as heterozygous for G-6-PD deficiency. Parasite counts have been made in both normal and enzyme-deficient cells and these data are now being analyzed.

Project 3A663713D829 MALARIA PROPHYLAXIS

Task 00, Malaria Investigations

Work Unit 112 Field studies on drug resistant malaria

Literature Cited.

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Publications:

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION ^a	2. DATE OF SUMMARY ^a	REPORT CONTROL SYMBOL	
				DA OA 6537	72 08 01	DD-DR&E(AR)636	
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72 07 01	H. TERM.	U	U	NA	NL	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	A. WORK UNIT
10. NO./CODES ^a		PROGRAM ELEMENT		PROJECT NUMBER		TASK AREA NUMBER	
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B. CONTRIBUTING							
C. CONTRIBUTING		CDOG 114 (F)					
11. TITLE (Precede with Security Classification Code) ^a							
(U) Malaria Program Supervision (09)							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS ^a							
012100 Organic Chemistry							
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
66 07		72 38		DA		C. In-House	
17. CONTRACT/GRANT				18. RESOURCES ESTIMATE			
A. DATES/EFFECTIVE: NA				PRECEDING			
B. NUMBER: ^a				FISCAL YEAR			
C. TYPE:				72			
D. KIND OF AWARD:				73			
E. CUM. AMT.				9			
19. RESPONSIBLE DOD ORGANIZATION				20. PERFORMING ORGANIZATION			
NAME: ^a Walter Reed Army Institute of Research				NAME: ^a Walter Reed Army Institute of Research			
ADDRESS: ^a Washington, D. C. 20012				ADDRESS: ^a Division of Medicinal Chemistry			
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21. GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER: [REDACTED]			
Foreign Intelligence Not Considered				ASSOCIATE INVESTIGATORS			
				NAME: Sweeney, Thomas R., Ph.D.			
				NAME: DA			
22. KEYWORDS (Precede EACH with Security Classification Code)							
(U) Malaria; (U) Drugs; (U) Biology; (U) Chemistry							
23. TECHNICAL OBJECTIVE, ^a 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
23. (U) To manage, to integrate, and provide quality control for the Drug Research Program on Malaria, both in-house and by contract.							
24. (U) To define areas requiring investigation, to develop suitable contract proposals to follow progress by correspondence or site visits, to guide direction of investigation, to provide for exchange of information, and to continually check findings for verification through independent agencies (both in-house and contract). Two outside advisory groups are utilized.							
25. (U) Studies formerly reported under this work unit are reported under 3A663713D829, Work Unit Number 134.							

^aAvailable to contractors upon originator's approval.

DD FORM 1498
1 MAR 58

PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 65 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE

PII Redacted

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION ^a	2. DATE OF SUMMARY ^a	REPORT CONTROL SYMBOL DD-DR&E(AR)636	
3. DATE PREV SUMMARY	4. KIND OF SUMMARY	5. SUMMARY SCTY ^a	6. WORK SECURITY ^a	7. REGRADING ^a	8A. DR&E ^a INSTN ^a	8B. SPECIFIC DATA- CONTRACTOR ACCESS	9. LEVEL OF DUN
72 07 01	H. TERM.	U	U	NA	NL	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	A. WORK UNIT
10. NO./CODES ^a	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER			
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B. CONTRIBUTING							
C. CONTRIBUTING	CDOG 114 (F)						
11. TITLE (Precede with Security Classification Code) ^a							
(U) Test system Design and Development							
12. SCIENTIFIC AND TECHNOLOGICAL AREA ^a							
002600 Biology							
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
70 07		72 08		DA		In-House	
17. CONTRACT/GRANT				18. RESOURCES ESTIMATE		19. PROFESSIONAL MAN YRS	
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B. NUMBER:				FISCAL YEAR		D. FUNDS (In thousands)	
C. TYPE:				CURRENT		105	
D. KIND OF AWARD:				73		105	
E. AMOUNT:				3		105	
F. CUM. AMT.				3		105	
19. RESPONSIBLE DOD ORGANIZATION				20. PERFORMING ORGANIZATION			
NAME: Walter Reed Army Institute of Research				NAME: Walter Reed Army Institute of Research			
ADDRESS: Washington, D. C. 20012				Division of Medicinal Chemistry			
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NAME: BUESCHER, E. L., COL				NAME: Kinnamon, K. E., COL			
TELEPHONE: 202/576-3551				TELEPHONE: 202/576-2292			
				SOCIAL SECURITY ACCOUNT NUMBER: [REDACTED]			
21. GENERAL USE				ASSOCIATE INVESTIGATORS			
Foreign Intelligence Not Considered				NAME: Smith, J. H., 1LT			
				NAME: Rich, R. R., 1LT			
				DA			
22. KEYWORDS (Precede EACH with Security Classification Code) (U) Chemical; (U) Chemistry; (U) Pharmaceutical;							
(U) Test System; (U) In Vitro; (U) In Vivo							
23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
23. (U) To design and develop in vitro and in vivo test systems for the evaluation of chemical compounds as prophylactic and therapeutic agents against diseases of major military significance. Infections such as malaria, which render troops ineffective for extended periods, receive principal emphasis.							
24. (U) Appropriate in vitro and animal pilot models are designed to meet a specific need for testing chemical compounds. These models are expanded into operating test systems in-house and utilized to provide guidance to contractors when testing is to be done under contract. When necessary, modifications to existing test systems are designed and developed.							
25. (U) Studies formerly reported under this work unit are reported under 3A663713D829, Work Unit Number 124.							

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION#	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL	
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(U) Biological studies on control of anopheline vectors of malaria							
12. SCIENTIFIC AND TECHNOLOGICAL AREA							
002600 Biology							
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
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NAME: Walter Reed Army Institute of Research				NAME: Walter Reed Army Institute of Research			
Washington, D.C. 20012				Div of CD and I			
ADDRESS:				ADDRESS: Washington, D.C. 20012			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Punish MAN if U.S. Academic Institution)			
NAME: Buescher, COL, E. L.				NAME: Ward, Dr. R.A.			
TELEPHONE: 202 - 576-3551				TELEPHONE: 202 - 576-2553			
				SOCIAL SECURITY ACCOUNT NUMBER: [REDACTED]			
21. GENERAL USE				ASSOCIATE INVESTIGATORS			
Foreign Intelligence Not Considered				NAME: Eldridge, LTC B.F.			
				NAME: Schiefer, MAJ B.A.			
				DA			
22. KEYWORDS (Precede EACH with Security Classification Code)							
(U) Anopheles; (U) Biocontrol; (U) Colonize; (U) Mosquitoes; (U) Vectors							
23. (U) Development of methods of the control of mosquito vectors of malaria which will have minimal impact upon the environment. Various mosquito pathogens will be studied to determine those types which can be utilized in a biological or integrated vector control program, which could decrease the incidence of malaria in OCONUS military personnel.							
24. (U) Establishment of laboratory colonies of anophelines from areas of strategic importance. Evaluation of rearing procedures through alteration of environmental factors. Study of mosquito pathogens on mosquito behavior, especially as related to disease transmission.							
25. (U) 72 07 - 72 08. Work formerly performed under this work unit now reported under Project 3A663713D829, Work Unit 124.							

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11. TITLE (Precede with Security Classification Code)							
(U) Biological Studies of mosquito malaria infection and transmission (09)							
12. SCIENTIFIC AND TECHNOLOGICAL AREA							
002600 Biology							
13. START DATE		14. ANTICIPATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
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f. CUM. AMT.						130	
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NAME: Walter Reed Army Institute of Research Washington, D.C. 20012				NAME: Walter Reed Army Institute of Research			
ADDRESS:				ADDRESS: Div of CD and I Washington, D.C. 20012			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Provide SSAN if U.S. Anomalous Information)			
NAME: Buescher, COL, E.L.				NAME: Eldridge, LTC B.F.			
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22. GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER: [REDACTED]			
Foreign Intelligence Not Considered				ASSOCIATE INVESTIGATORS			
				NAME: Ward, Dr. R.A.			
				NAME: Schneider, Dr. I.			
				DA			
23. REVISIONS (Precede with Security Classification Code)							
(U) Anopheles; (U) Mosquitoes; (U) Plasmodium;							
(U) Susceptibility; (U) Falciparum malaria; (U) Aotus; (U) Immunization							
24. TECHNICAL OBJECTIVE, 25. APPROACH, 26. PROGRESS (Provide individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
<p>23. (U) Development of physiological means of interrupting malaria transmission through an understanding of factors influencing susceptibility of anopheline vectors to malaria and of factors determining transmission efficiency of vectors. Test systems are developed for the evaluation of antimalarial drugs and the study of sporozoite immunity.</p> <p>24. (U) Studies are conducted on the infectivity of human and closely related simian malarial parasites to various mosquito vectors of SE Asian areas. Attempts to transmit falciparum malaria to lower primates are made. Feedings of anophelines on gametocytic hosts are conducted, followed by dissections of samples of the mosquitoes at intervals thereafter to determine level and progress of infections.</p> <p>25. (U) 72 07 - 73 06. Aotus monkeys treated with methionine and inoculated with malaria sporozoites from infected Anopheles stephensi showed evidence of infection after 18 days of patency. Non-methionine treated controls remained negative. Fine structure differences mainly in the gametocyte, were observed between a chloroquine-resistant (Smith) strain and a chloroquine-susceptible strain of malarial parasite. Anopheles stephensi mosquitoes infected with Plasmodium cynomolgi flew shorter distances and more slowly on a mechanical flight mill than did non-infected controls. Using density gradients, infectious sporozoites were isolated from homogenates of infected anopheline mosquitoes. Diploid cell lines of Armigeres subalbatus and Aedes atropalpus were established. Work reported under this unit was formerly reported under Project 3A663713D829, Work Units 123, 125, and 126. For technical reports see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 72 - 30 June 73.</p>							

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Project 3A663713D829 MALARIA PROPHYLAXIS

Task 00 Malaria Investigations

Work Unit 124 Biological studies of mosquito malaria infection and transmission

Investigators

Principal: LTC Bruce F. Eldridge, MSC; Ronald A. Ward, Ph.D.;
Imogene Schneider, Ph.D.

Associate: MAJ Bernard A. Schiefer, MSC; CPT Anthony Bosworth, MSC;
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SP4 David Spooner; SP4 John Brandt

Description

Studies are conducted on the relationship of human and non-human malarial parasites to their mosquito vectors. Included are consideration of the role of genetic and environmental factors in mosquito susceptibility, the effect of mosquito pathogens on mosquito biology, the design of insect culture systems which will support the growth and development of the insect phase of malarial parasites, the use of density gradients to isolate sporozoites from mosquitoes, and the development of primary and established insect cell lines from selected mosquito species.

Progress

1. Mosquito transmission of Plasmodium falciparum in monkeys.

Although anopheline mosquitoes can be infected with falciparum malaria from Aotus trivirgatus donors, and the complete sporogonous cycle shown by transmission back to man or chimpanzee, monkey to monkey transmission by sporozoites has only been achieved once (3). The repeated transmission of this parasite by mosquitoes has obvious implications for the control of malaria.

Sodeman et al. (9) observed partial development of the exoerythrocytic (EE) schizont of this parasite in Aotus. However, the EE schizonts strained abnormally and did not release infective merozoites. They thought that the hepatic cell of Aotus lacked a necessary metabolite for the maturation of this stage. Pathological examination of liver tissues from captive Aotus which had not been experimentally infected with malaria showed focal degenerative changes such as advanced fatty livers or hydropic degeneration in 2/6 animals (5). These conditions are often a consequence of a dietary deficiency and may be reversed with the administration of essential amino acids. It was inferred that the inability of Aotus to sustain a complete EE parasite cycle was related to an unfavorable hepatic environment, and that this might be reversed by dosage of Aotus with various essential amino acids that were known to be of value in treating certain forms of fatty liver in experimental animals. Methionine was the first such amino acid to be considered.

Two splenectomized Aotus trivirgatus were inoculated intravenously with P. falciparum (Vietnam, Smith strain) sporozoites from Anopheles stephensi mosquitoes fed on Aotus donors. 50-70 infected salivary glands were dissected in chilled Mosquito Culture Medium (Grand Island Biological Co.), triturated and injected into the femoral vein in 1 ml. of solution. One Aotus was treated with methionine and the other served as a control. 0.2 g. of dimethionine was administered orally 4 days prior to the sporozoite inoculation and 5 times weekly for the following 30 days. The methionine-treated animal exhibited P. falciparum trophozoites in the peripheral circulation 18 days after sporozoite passage while the control Aotus was negative over a 56 day observation period. Immature and mature falciparum gametocytes appeared 11 and 20 days respectively, after patency in the treated Aotus, thus confirming the identity of the transmission. These gametocytes subsequently proved infective to Anopheles stephensi.

This transmission is of interest as the prepatent period of the Smith strain was 18 days as contrasted to the 36 day period observed by Collins and Contacos (3) with the Cambodian I strain in this host. It is hypothesized that the present circumstances of transmission to a methionine-treated animal indicate the metabolic state of the liver may be an important factor in sustaining complete EE development of certain P. falciparum strains in abnormal host systems.

2. Fine structure of Plasmodium falciparum (Vietnam, Smith strain).

This strain of falciparum malaria is highly resistant to chloroquine treatment both in man and the Aotus monkey. In collaboration with M. Aikawa (Case Western Reserve University) the fine structure of the Smith strain was examined in order to determine whether there is any structural difference between chloroquine sensitive and resistant strains of P. falciparum.

Striking morphological differences between these two strains are observed mainly in the gametocyte. The gametocytes of the chloroquine sensitive strain of P. falciparum show two membranes surrounding the parasite, while those of the Smith strain show three membranes. A row of microtubules is seen just beneath the membrane layer and follows the limiting membrane of the parasite, while microtubules are rarely observed in the chloroquine sensitive gametocytes. This is true of other species of plasmodial gametocytes.

3. The Effect of Plasmodium cynomolgi on the flight of Anopheles stephensi Liston

The degree of physiological damage to mosquitoes by developing malarial parasites has not been clearly determined. It is recognized that the plasmodial infection has the potential of causing some damage at several stages during its development in the mosquito host. Initial penetration of the ookinetes through the stomach wall, development and

rupture of oocysts, and invasion of tissue by migrating sporozoites might be expected to have some mechanical, pathological or other deleterious effect on the mosquito.

Several workers (1,6) have compared the longevity and vitality of infected and uninfected mosquitoes and concluded that there are no apparent detrimental effects by the plasmodia. Conversely, many others have suggested that plasmodial infections may harm the mosquito (2,8). De Buck & Swellengrebel (4) found greater mortality among heavily infected mosquitoes in relation to lightly infected ones. They are of the opinion that salivary infections are harmful while intestinal infections alone were not.

A reduction in flight range of infected mosquitoes and its relation to the localized nature of some malaria epidemics has been questioned (8). A reduction in mosquito flight ability due to malaria infection would have important epidemiological implications; therefore, this experiment was designed to determine the effect of Plasmodium cynomolgi on the flight on Anopheles stephensi at two critical points during parasite development.

Mosquitoes of the "India strain" of Anopheles stephensi Liston maintained at the Walter Reed Army Institute of Research since 1962 were used. They were reared in small pans (23 x 39 x 6 cm) on a precise timetable so that development day 7 pupae were exclusively used. Each day, approximately 200 female pupae were placed in a pint carton with a screened top. The water was drained off after emergence and they were held at $25.6\text{ C} \pm 0.16$ and $80.9\% \pm 0.21$ relative humidity with a 14 hour photoperiod. Cotton pads saturated with 10% sucrose solution were available to the mosquitoes.

Plasmodium cynomolgi Mayer of the "B" strain (P. c. bastianellii) was maintained in rhesus monkeys by either mosquito or blood induction. Quantitative determination of circulating parasitemia levels were made at time of each mosquito feeding period.

Mosquitoes were fed on infected or non-infected monkeys on day 4 and flown 12 days later or fed on day 10 and flown 6 days later so that all mosquitoes were 16 days old when flown. These periods were selected to correspond to the period of maximum maturity of oocyst and salivary gland infection. Due to low oocyst development and low weights of mosquitoes feeding on day 10 a second comparison was made by feeding mosquitoes on day 4 and flying 6 days later. During this portion of the study, a single lot of mosquitoes was divided with 1/2 feeding on an infected monkey and the remainder on a non-infected monkey. Four mosquitoes from each group were flown at the same time on day 10, paired by weight, and analyzed by differences of paired comparisons. After feeding, mosquitoes were anesthetized with ether and 50 engorged mosquitoes were retained for later flight. Eight mosquitoes from each lot held for 12 days post-feeding were dissected on day 7 for oocyst counts.

Mosquitoes were flown on flight mills in the manner previously described by Schiefer et al, (7). Eight mosquitoes were flown daily until a total of 80 lots had been flown which provided approximately 50 mosquitoes for each category of comparison. Mosquitoes were weighed prior to attachment to the flight mill arm and flown for a 24 hour period in continuous light at an average temperature of 23.13 ± 0.21 S.E. and a relative humidity of $59.95\% \pm 0.88$ S.E. Mosquitoes weighing between 2.0 and 3.0 mg were selected for flight to minimize the effect of weight variability. Flight distance, duration and number of flights were recorded after 24 hours and the mosquitoes were re-weighed to determine weight lost. Mosquitoes were then dissected to determine degree of gravidity, sporozoite infection and/or number of oocysts.

Preliminary data indicates that the development of malarial parasites in the mosquito causes some reduction in the flight ability of Anopheles stephensi (Table 1). The distance flown by 16 day old mosquitoes fed on day 4 on a non-infected monkey was 6.363 km. This was statistically greater than the same age mosquitoes fed on infected monkeys and which developed sporozoites (4.385 km) or which had mature oocysts but without salivary gland infection (4.437 km). Mosquitoes which fed on infected monkeys but where no parasites were found or which did not develop flew an average of 5.493 km.

A paired comparison of 10 day old mosquitoes fed on day 4 showed consistent decreased flight distance compared to their paired controls. The mean paired difference was -1.343 km. An analysis of the differences by the "t" test showed significance at the 1% level of probability. The flight distance of mosquitoes which fed on infected monkeys but which developed no parasites or whose parasites were not detected was not different from their paired controls.

Although the reduction in flight ability is not severe, it is clearly evident in both categories of mosquitoes examined.

4. Cultivation of malaria parasites and mosquito tissues

Plasmodium cynomolgi oocysts of varying ages were placed in culture with cells from primary cultures as well as from an established cell line of Anopheles stephensi. Four day old oocysts showed the least favorable response to in vitro culture, whereas 6 day old oocysts responded primarily by growth and 8 day old oocysts by differentiation. There were no striking differences in the response of P. cynomolgi oocysts to cells of the established line versus those from the primary culture. Cells from adult tissue were no more effective than those from larval tissues in promoting development of the parasites in vitro. However, the oocysts invariably did better when cultured together with A. stephensi cells, regardless of origin, than in medium alone.

Infected Rhesus monkey blood, having a high gametocyte count, was placed in culture with A. stephensi cells. Although some exflagellation

was observed shortly after the blood was introduced, few ookinetes were found after the preparations were fixed and stained at 24 or 48 hours.

Attempts are currently being made to grow A. stephensi mosquitoes axenically using media designed by Rosales-Ronquillo, Nayar and Lang. Assuming these attempts are successful, primary cultures from midgut cells will be initiated and various stages of P. cynomolgi introduced into them.

5. Isolation of malaria sporozoites on density gradients and their subsequent use in the attempted immunization of Rhesus monkeys

The main obstacle in isolating P. cynomolgi sporozoites from homogenized A. stephensi mosquitoes on density gradients has been the osmotic fragility of the parasites. This problem was resolved during the past year by employing in the gradient "Path-o-cyte" bovine albumin solutions which are adjusted with respect to pH, specific gravity and salinity. Sporozoites isolated from the gradient were inoculated IV into a number of Rhesus monkeys and the prepatent period as well as the parasitemia was comparable to that of the controls.

Prior work by Nussenzweig et al., has demonstrated that killed P. berghei sporozoites, when used as antigens, did not give protection against rodent malaria whereas the use of sporozoites attenuated by X-irradiation did. This suggests that the products of sporozoite metabolism rather than the sporozoites themselves serve as a more potent antigenic stimulus. A successful demonstration of such potency would have obvious implications in the development of an antimalarial vaccine.

Control and infected A. stephensi mosquitoes were irradiated with 9 kilorads in a "Gammacell 220" cobalt source. The mosquitoes were exposed in pint-sized cardboard containers at a dose rate of approximately 7290 rads per minute. After irradiation, both control and infected mosquitoes were subjected to homogenization and comparable fractions placed on separate density gradients. Sporozoites recovered from the peak fractions of the gradient were washed with a buffered salt solution and 0.5 - 1.0 ml injected IV into each of two monkeys. The control monkey was injected IV with the material from comparable fractions of the control gradient. Each monkey received a total of four injections spaced at bi-weekly intervals. The numbers of inoculated sporozoites varied from a low of 2×10^4 to a high of 6.5×10^6 and totaled approximately 10^7 per experimental monkey. Two weeks after the final injection, six to ten infected mosquitoes were allowed to feed on each of the three monkeys. The prepatent period for both the control and experimentals was 9-10 days. The parasitemia curves, at least for the initial cycle, appear to be quite similar for all three monkeys.

A second series of immunizations are currently being carried out. The procedure is identical except for the spacing of injections. Two immunizing doses (approximately 10^6 sporozoites per animal) have been given four days apart and will be followed by a third injection one month later.

6. Establishment of cell lines from various mosquito species and other dipterans

In anticipation of using the cells for comparative studies involving penetration and replication of mosquito iridescent virus in vitro, cell lines of Armigeres subalbatus and Aedes atropalpus were initiated. In both instances the primary cultures consisted of minced, trypsinized first stage larvae. Subculturing was successful within ten days after the cultures had been set up. The lines are now in the 11th and 12th passages, respectively.

An incipient cell line of Glossina morsitans has also been initiated. These cells will be used in an attempt to study the metabolism of the crithidial and metacyclic forms of several species of African trypanosomes.

Conclusions and recommendations

1. Cyclical transmission of the Vietnam, Smith strain of falciparum malaria was demonstrated in the Aotus monkey. It is believed that the metabolic condition of the host liver is an important factor in the establishment of an exoerythrocytic cycle in an abnormal host. Further studies should be conducted with various essential amino acids to determine their role in the development of this malarial parasite.

2. The distinct morphological differences between the gametocytes of the chloroquine sensitive and resistant strains of P. falciparum indicate that the resistant strains are morphologically and physiologically altered. The presence of microtubules in the Smith strain may indicate that they may play a role in the development of chloroquine resistance.

3. Anopheles stephensi mosquitoes infected with Plasmodium cynomolgi flew shorter distances and more slowly on mechanical flight mills than did non-infected controls.

4. Attempts to grow the mosquito phases of malaria parasites in vitro should be temporarily suspended and efforts directed towards growing the anopheline vector aseptically. Once this is accomplished it should be possible to initiate cultures from midgut cells. These may prove to be a more adequate substrate for the growth and development of the parasites than the multiple cell type cultures which have been used in the past.

5. The isolation of fully infectious P. cynomolgi sporozoites from homogenates of infected A. stephensi mosquitoes on density gradients has been realized. When injected into Rhesus monkeys both the prepatent periods and the levels of parasitemia are comparable to that of the controls. The resulting preparations are still, however, contaminated with some bacteria and efforts should be made to eliminate them. Efforts should also be made to increase the yield of sporozoites from any one gradient and to cycle the parasite between monkey and mosquito with

grater frequency. If this is accomplished it should be possible to increase the number of sporozoites to 10^7 for both the initial and the booster innocations. Further variations in the spacing of the injections should also be explored.

TABLE 1

Parameter values associated with 24 hour flight of 191 sixteen day old An. stephensi mosquitoes after 12 days post blood feed

Host	Infection category	Infective rhesus				Non-infected rhesus			
		Sporozoites present		Oocysts present no sporozoites		No infection found/developed		Control	
		Mean	S.E.	Mean	S.E.	Mean	S.E.	Mean	S.E.
Distance flown (km)		4.385	± 0.284	4.522	± 0.406	5.493	± 0.373	6.363	± 0.375
Total flight duration (min)		257.782	± 17.701	268.290	± 22.904	293.978	± 17.607	317.644	± 16.352
Speed of flight (m/min)		17.552	± 0.514	17.003	± 0.674	18.926	± 0.696	19.706	± 0.503
Length of initial flight (min)		42.982	± 8.893	32.323	± 8.038	58.609	± 12.816	90.678	± 15.467
Length of longest flight (min)		93.945	± 10.374	90.645	± 13.214	120.174	± 14.114	172.186	± 14.415
Number of flights (#/24hr)		36.073	± 2.801	41.290	± 4.348	36.848	± 3.251	33.356	± 2.666
Pre flight weight		2.276	± 0.034	2.312	± 0.050	2.332	± 0.035	2.520	± 0.032
Post flight weight		1.674	± 0.032	1.726	± 0.048	1.612	± 0.029	1.794	± 0.035
Number flown		55		31		46		59	

** significant at 1% level.

Project 3A663713D829 MALARIA PROPHYLAXIS

Task 00 Malaria Investigations

Work Unit 124 Biological studies of mosquito malaria infection and transmission

Literature Cited.

References:

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7. Schiefer, B.A., Williams, J., Neal, T.J., and Eldridge, B.F.: Laboratory flight studies with Anopheles stephensi Liston (Diptera: Culicidae). J. Med. Entomol., In press.
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1. Rutledge, L.C., Ward, R.A., and Hayes, D.E.: Plasmodium cynomolgi: The comparative infectiousness of individual rhesus monkeys. Expt. Parasitol. 33:120-126, 1973.
2. Schneider, I.: A comparative study of the development of the mosquito cycle of Plasmodium cynomolgi in primary cultures versus an established cell line of Anopheles stephensi. Proc. Helmin. Soc. Wash. 39:438-444, 1972.
3. Schneider, I.: Establishment of cell lines from Culex tritaeniorhynchus and Culex salinarius (Diptera: Culicidae). Proc. III International Colloq. Invertebrate Tissue Culture, Slovak Academy of Science, Bratislava, Czechoslovakia. pp. 121-134, 1973.
4. Ward, R.A., and Hayes, D.E.: Sporozoite transmission of Falciparum malaria (Vietnam, Smith strain) from monkey to monkey. Trans. Roy. Soc. Trop. Med. Hyg. 66:670-671, 1972.
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6. Ward, R.A., Hayes, D.E., Hembree, S.C., Rutledge, L.C., Anderson, S.J., and Johnson, A.J.: Infectivity of Plasmodium falciparum gametocytes from Aotus trivirgatus to anopheline mosquitoes. Proc. Helminthol. Soc. Wash. (Special issue) 39:33-46, 1972.
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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION ^a	2. DATE OF SUMMARY ^a	REPORT CONTROL SYMBOL DD-DR&E(AR)636	
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11. NO./CODES ^a		PROGRAM ELEMENT		PROJECT NUMBER		TASK AREA NUMBER	
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B. CONTRIBUTING						125	
C. CONTRIBUTING		CD06114(F)					
11. TITLE (Precede with Security Classification Code) ^a							
(U) Taxonomy and Ecology of Disease Bearing Mosquitoes of Southeast Asia (09)							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS ^a							
002600 Biology							
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65 07		72 08		DA		C. In-House	
17. CONTRACT/GRANT				18. RESOURCES ESTIMATE		A. PROFESSIONAL MAN YRS	
A. DATES/EFFECTIVE ^a Not Applicable				PRECEDING		B. FUNDS (in thousands)	
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F. CUM. AMT.							
19. RESPONSIBLE DOD ORGANIZATION				20. PERFORMING ORGANIZATION			
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				NAME: Harrison, CPT, B.A.			
				DA			
22. KEYWORDS (Precede EACH with Security Classification Code) (U) Anopheles; (U) Aedes; (U) Mosquitoes; (U) Malaria; (U) Arboviruses; (U) Disease Vectors							
23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number Precede text of each with Security Classification Code.)							
23. (U) To produce keys, guides, and other guides for identification of mosquitoes which are vectors of diseases of military importance. Emphasis is on vectors of malaria and arbovirus diseases such as dengue. Also, to obtain data on the ecology, biology, and disease transmission potential of these mosquitoes.							
24. (U) Mosquitoes are collected by military and civilian cooperating agencies and are forwarded to a joint WRAIR-Smithsonian Institution team for identification. This team intensively studies these collections, as well as collections in established museums, and publishes keys, guides, and other identification aids which are returned to SE Asia for use by entomologists engaged in control and survey operations. Team also accumulates and makes available disease transmission and biological data.							
25. (U) 72 07 - 72 08. Work formerly performed under this work unit now reported under Project 3A663713D829, Work Unit 124.							

^a Available to contractors upon originator's approval

DD FORM 1498
1 MAR 66

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AND 1498-1, 1 MAR 66 (FOR ARMY USE) ARE OBSOLETE

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION ^a	2. DATE OF SUMMARY ^a	REPORT CONTROL SYMBOL DD-DR&E(AR)636	
3. DATE PREV SUMMARY 72 07 01	4. KIND OF SUMMARY H. Term.	5. SUMMARY SCTY ^a U	6. WORK SECURITY ^a U	7. REGRADING ^a NA	8. DISSEM INSTR ^a NL	9a. SPECIFIC DATA- CONTRACTOR ACCESS <input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	9. LEVEL OF SUM A. WORK UNIT
10. NO./CODES: ^a		PROGRAM ELEMENT		PROJECT NUMBER		TASK AREA NUMBER	
a. PRIMARY		63713A		3A663713D829		00	
b. CONTRIBUTING						126	
c. XXXXXXXX		CD0G114(F)					
11. TITLE (Precede with Security Classification Code) ^a							
(U) In Vitro Cultivation of Mosquito Tissues and Malarial Parasites (09)							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS ^a							
002600 Biology							
13. START DATE 65 07		14. ESTIMATED COMPLETION DATE 72 08		15. FUNDING AGENCY DA		16. PERFORMANCE METHOD C. In-House	
17. CONTRACT/GRANT				18. RESOURCES ESTIMATE ^a		19. PROFESSIONAL MAN YRS	
a. DATES/EFFECTIVE: Not Applicable				PRECEDING			
b. NUMBER: ^a				FISCAL YEAR		b. FUNDS (in thousands)	
c. TYPE:				72		2	
d. KIND OF AWARD:				73		30	
e. AMOUNT:							
f. CUM. AMT.							
20. RESPONSIBLE DOD ORGANIZATION				20. PERFORMING ORGANIZATION			
NAME: ^a Walter Reed Army Institute of Research Washington, D.C. 20012				NAME: ^a Walter Reed Army Institute of Research Div of CD and I			
ADDRESS: ^a				ADDRESS: ^a Washington, D.C. 20012			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)			
NAME: Buescher, COL E. L.				NAME: ^a Schneider, I. Ph.D.			
TELEPHONE: 202 - 576-3551				TELEPHONE: 202 - 576-3049			
21. GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER: [REDACTED]			
Foreign Intelligence Not Considered				ASSOCIATE INVESTIGATORS			
				NAME: DA			
22. KEYWORDS (Precede EACH with Security Classification Code) (U) Aedes; (U) Anopheles; (U) Culex (U) Mosquitoes; (U) Malaria; (U) Tissue Culture; (U) Immunology							
23. TECHNICAL OBJECTIVE, ^a 24. APPROACH, 25. PROGRAM (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
23. (U) To develop reliable in vitro procedures by which large quantities of relatively pure malarial sporozoites can be produced for malaria vaccine development studies. Also, to develop mosquito tissue culture systems for studies on pathogen invasion and growth dynamics in invertebrate tissue.							
24. (U) Development of culture media which will support growth of invertebrate stages of malaria parasites. Development of various techniques for the isolation and purification of individual stages of parasite. Evaluation of mosquito life cycle stages for suitability for establishing primary cultures.							
25. (U) 72 07 - 72 08. Work formerly performed under this work unit now reported under Project 3A663713D829, Work Unit 124.							

^a Available to contractors upon originator's approval

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1 MAR 68

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1141

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION ^a	2. DATE OF SUMMARY ^a	REPORT CONTROL SYMBOL
				DA OA 6518	72 08 01	DD-DR&E(AR)636
3. DATE PREV SUMMARY	4. KIND OF SUMMARY	5. SUMMARY SCTY ^a	6. WORK SECURITY ^a	7. REGRADING ^a	8A. DR&E INSTR ^a	8B. SPECIFIC DATA- CONTRACTOR ACCESS
72 07 01	H, TERMINATION	U	U	NA	NL	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO
9. NO./CODES ^a	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER		
a. PRIMARY	63713A	3A663713D829	00	127		
b. CONTRIBUTING						
c. CONTRIBUTING	CDOG 114 (F)					
11. TITLE (Precede with Security Classification Code) ^a						
(U) Test Systems for Plasmodium Falciparum (09)						
12. SCIENTIFIC AND TECHNOLOGICAL AREA ^a						
000000 Biology						
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD
65 07		72 08		DA		D, IN-House
17. CONTRACT/GRANT				18. RESOURCES ESTIMATE		19. PROFESSIONAL MAN YRS
NA				PRECEDING		2. FUNDS (in thousands)
a. DATES/EFFECTIVE:		EXPIRATION:		FISCAL YEAR	72	70
b. NUMBER:		c. TYPE:		CURRENT	73	70
d. AMOUNT:		e. CUM. AMT:				
19. RESPONSIBLE DOD ORGANIZATION				20. PERFORMING ORGANIZATION		
NAME: Walter Reed Army Institute of Research				NAME: Walter Reed Army Institute of Research		
ADDRESS: Washington, D.C. 20012				Division of CD&I		
				ADDRESS: Washington, D.C. 20012		
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)		
NAME: BUESCHER, COL. E. L.				NAME: SADUN, E. H., Sc.D.		
TELEPHONE: 202-576-3551				TELEPHONE: 202-576-3308		
				SOCIAL SECURITY ACCOUNT NUMBER: [REDACTED]		
21. GENERAL USE				ASSOCIATE INVESTIGATORS		
Foreign Intelligence Not Considered				NAME: MOON, A.P. DA		
				NAME:		
22. KEYWORDS (Precede EACH with Security Classification Code) ^a						
(U) Immunity; (U) Chloroquine; (U) Gamma Globulin;						
(U) Isotope; (U) Susceptibility; (U) Owl Monkey						
23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)						
23 (U) Study susceptibility of chimpanzees and other primates to infections of human malaria. Study the characteristics of drug resistant strains, provide high density of parasites for morphological and biochemical studies. Conduct physiological and pathological studies of malaria and provide test animals for chemotherapeutic and immunological investigations.						
24 (U) Infect splenectomized, drug treated chimpanzees and other primates with plasmodia of human origin. Observe the extent and duration of parasitemias, study and response of different strains to chemotherapy, study susceptibility to reinfection with homologous and heterologous strains.						
25 (U) 72 07 - 73 08 Work unit is being combined with work unit number 129, line item 3A663713D829.						

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION ^a	2. DATE OF SUMMARY ^a	REPORT CONTROL SYMBOL	
				DA OA 6519	72 08 01	DD-DR&E(AR)636	
3. DATE PREV SUMMARY	4. KIND OF SUMMARY	5. SUMMARY SCTY ^a	6. WORK SECURITY ^a	7. REGRADING ^a	8. DR&E NSTR ^a	9. SPECIFIC DATA - CONTRACTOR ACCESS ^a	10. LEVEL OF SUB A. WORK UNIT
72 07 01	H. TERMINATION	U	U	NA	NL	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	
11. NO./CODES: ^a		PROGRAM ELEMENT		PROJECT NUMBER		TASK AR. A NUMBER	
a. PRIMARY		63713A		3A663713D829		70	
b. CONTRIBUTING						128	
c. CONTRIBUTING		CDOG 114 (F)					
11. TITLE (Precede with Security Classification Code) ^a							
(U) Natural and Acquired Immunity in Rodent Malaria (09)							
12. SCIENTIFIC AND TECHNOLOGICAL AREA ^a							
002600 Biology							
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
65 07		72 08		DA		C. IN-HOUSE	
17. CONTRACT/GRANT NA				18. RESOURCE: ESTIMATE		a. PROFESSIONAL MAN YRS	
a. DATES/EFFECTIVE:				PRECEDING		b. FUNDS (in thousands)	
b. NUMBER: ^a				FISCAL YEAR		70	
c. TYPE:				CURRENT		70	
d. KIND OF AWARD:				73		2	
e. CUM. AMT.							
19. RESPONSIBLE DOD ORGANIZATION				20. PERFORMING ORGANIZATION			
NAME: ^a Walter Reed Army Institute of Research				NAME: ^a Walter Reed Army Institute of Research			
ADDRESS: ^a Washington, D.C. 20312				Division of CD&I			
				ADDRESS: ^a Washington, D.C. 20012			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)			
NAME: BUESCHER, COL. E. L.				NAME: ^a SADUN, E. H., Sc.D.			
TELEPHONE: 202-576-3551				TELEPHONE: 202-576-3308			
				SOCIAL SECURITY ACCOUNT NUMBER: [REDACTED]			
21. GENERAL USE				ASSOCIATE INVESTIGATORS			
Foreign Intelligence Not Considered				NAME: MOON, A. P. DA			
				NAME:			
22. KEYWORDS (Precede EACH with Security Classification Code) (U) Rodents; (U) Susceptibility; (U) Immunity; (U) Plasmodium; (U) Irradiate							
23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
23 (U) To evaluate the role of humoral and cellular factors in determining susceptibility of hosts to malaria, for the maintenance of the complete life cycle of malaria in the laboratory, to find a laboratory animal suitable for the production of large amounts of infected blood for immunological and biochemical studies.							
24 (U) To test a variety of rodent species for natural susceptibility to P. berghei. Attempts to increase susceptibility by splenectomy and chemical treatment. Standardize the course of infections quantitatively. Evaluate the mechanism of antibody action on host and parasite, and characterize antibodies responsible for these activities. Study the effects of antibody on the parasite and on the host.							
25 (U) 72 07 - 73 06 Work unit is being combined with work unit number 129, line item 3A663713D829.							

^a Available to contractors upon originator's approval.

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PII Redacted

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION ^a	2. DATE OF SUMMARY ^a	REPORT CONTROL SYMBOL DD-DR&E(AR)636	
3. DATE PREV SUMRY 72 07 01	4. KIND OF SUMMARY D. CHANGE	5. SUMMARY SCTY ^a U	6. WORK SECURITY ^a U	7. REGRADING ^a NA	8. ORIGIN INSTN ^a NL	9. SPECIFIC DATA - CONTRACTOR ACCESS <input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	10. LEVEL OF SUM A. WORK UNIT
11. NO /CODES: ^a	PROGRAM ELEMENT	PROJECT NUMBER		TASK AREA NUMBER		WORK UNIT NUMBER	
A. PRIMARY	63713A	3A663713D829		00		129	
B. CONTRIBUTING							
C. CONTRIBUTING	CDOG 114 (F)						
11. TITLE (Precede with Security Classification Code) ^a (U) Host Responses to Malaria (09)							
12. SCIENTIFIC AND TECHNOLOGICAL AREA ^a 002600 Biology							
13. START DATE 64 07		14. ESTIMATED COMPLETION DATE CONT		15. FUNDING AGENCY DA		16. PERFORMANCE METHOD In-House	
17. CONTRACT/GRANT A. DATES/EFFECTIVE: NA B. NUMBER: ^a C. TYPE: D. KIND OF AWARD:		EXPIRATION: E. AMOUNT: F. CUM. AMT.		18. RESOURCES ESTIMATE PRECEDING FISCAL YEAR 73 CURRENT 74		A. PROFESSIONAL MAN YRS 2 6	
						B. FUNDS (in thousands) 70 210	
19. RESPONSIBLE DOD ORGANIZATION				20. PERFORMING ORGANIZATION			
NAME: ^a Walter Reed Army Institute of Research ADDRESS: ^a Washington, D.C. 20012 RESPONSIBLE INDIVIDUAL NAME: BUESCHER, COL E. L. TELEPHONE: 202-576-3551				NAME: ^a Walter Reed Army Institute of Research Division of CD&I ADDRESS: ^a Washington, D.C. 20012 PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution) NAME: ^a SADUN, E. H., Sc.D. TELEPHONE: 202-576-3308 SOCIAL SECURITY ACCOUNT NUMBER: [REDACTED]			
21. GENERAL USE Foreign intelligence not considered				ASSOCIATE INVESTIGATORS NAME: MOON, A. P. DA NAME:			
22. KEYWORDS (Precede EACH with Security Classification Code) (U) Malaria; (U) Gamma globulin; (U) Biochemistry; (U) Antibody; (U) Fluorescent; (U) Isotope; (U) Metabolism							
23. TECHNICAL OBJECTIVE, ^a 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.) 23(U) To study the physiological pathology of malaria, including the enhancement of non-specific resistance to infection and to determine how energy requirements are met within the parasite. 24(U) Study the effect of infection on the uptake and distribution of radioisotope-labelled amino acids, study the levels of enzyme activity in tissue extracts and alterations in protein and free amino acid constituents of blood and urine, study the development of relapses, and the pattern of parasitemias and fluorescent antibodies prior to, during, and following therapy. Investigate the use of immune gamma globulins as an adjuvant to chemotherapy in humans infected with drug resistant malaria. 25(U) 72 07 - 73 06 A method for freezing and storing human erythrocytes for transfusions has been applied to storing erythrocytes containing Plasmodium falciparum from infected owl monkeys. The cells retain both the capacity to incorporate C-14 isoleucine into protein and to infect animals after storage. Changes in the capacity to synthesize protein and to infect animals were not detected during serial experiments over a seven week period. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 72 - 30 June 73.							

^a Available to contractors upon originator's approval.

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PII Redacted

Project 3A663713D829 MALARIA PROPHYLAXIS

Task 00 Malaria Investigations

Work Unit 129 Host responses to malaria

Investigators.

Principal: E.H. Sadun, Sc.D., Lib. Doc.

Associate: LTC C.L. Diggs, MC; S-5 K. Joseph; MAJ L.K. Martin, MSC; Sp-5 H.R. Snodgrass; CPT R.A. Wells, MSC

1. Approaches to the study of acquired immunity to malaria.

Acquired immunity to infectious disease is often considered to connote complete protection of an otherwise susceptible host from the disease in question. However, analysis of the immune response to certain pathogens is aided by consideration of the quantitative character of immunity. From this point of view absolute immunity simply refers to a situation in which the host has such a high degree of acquired resistance to a given pathogen that evidence of infection never arises at all. Partial immunity can be manifested as complete protection of some, but not all, of the individuals in a population, or as a state in which parasite growth and development occurs in the immune individual, but without fulminant disease. Nowhere is the concept of partial immunity more useful than in the case of the malarias; although immunity has been shown to be operative in many of these diseases, in almost every case it is partial. The reasons for this fact are poorly understood, but some insight may be gleaned from the considerations to follow.

In the course of attempts to interpret some of the unusual features of malaria, two specialized terms have evolved, which should be dealt with at the outset. The term "premunition" has been used for many years to describe a state in which immunity depends on persistent infection. It stems from the observation that individuals often sustain low grade parasitemia for long periods of time in an environment in which non-immune individuals develop acute illness with heavy parasitemia. Although the observations are undisputed, many observers question the dependence of immunity on chronic infection. In at least some host-parasite systems, immunity can be demonstrated in the absence of infection, a situation which has been termed "sterile immunity." Another term often used in describing immunity to malaria which is confusing to the general immunologist is "tolerance." It refers not to immunological unresponsiveness, but rather to the ability of the host to tolerate malaria without serious morbidity once the immune response is initiated. In areas of high endemicity, it is not unusual for patients with malignant tertian malaria to have a relatively high percentage of their erythrocytes parasitized without serious illness, whereas a nonimmune individual experiencing his first attack by the same parasite might be desperately ill with the same level of parasitemia.

In addition to studies on the unusual features of malaria, attempts have been made to relate phenomena observed in malaria with principles already documented in other infections. Thus, experiments on the passive transfer of immunity with serum and with cells have been performed and have been quite informative. In the presentation to follow both types of approaches will be kept in mind since both have important roles for increasing understanding of these complex phenomena.

The life cycle of malaria parasites includes several stages which theoretically could be attacked by immune mechanisms. Thus, the sporozoite, on entering the skin of the vertebrate host is presumably vulnerable to the phagocytes and other cells as well as to humoral factors in its transit to its intracellular site. In avian malaria parasites this may be in a cell of the mononuclear phagocyte series. It is thus possible that cellular immune mechanisms are induced, on exposure to the parasite antigens, to activate these mononuclear cells for more efficient phagocytosis and intracellular destruction of the parasites. It is in this situation, where the cells of the system of phagocytes serve as host cells for parasites, that cellular immune mechanisms seem less likely in the case of mammalian malarias where the site of first intracellular residence is the hepatocyte.

Following the tissue or exoerythrocytic stage, the parasites again are exposed to the extracellular milieu as merozoites which, if they are not inactivated, infect the erythrocytes. It would seem that the parasites might occupy a privileged position with respect to immunologic attack within the erythrocytes. However, as will be discussed below, the parasite may be the instrument of its own destruction in terms of alterations of the host cell during its development. At any rate, the parasite must, in order to survive, again enter the extracellular space during its transit to a new host cell. It is at this point that it is perhaps most vulnerable to host defense mechanisms, and it is this form (the merozoite) which many investigators believe is a prime target cell of immune effector activity.

II. General methods in experimental malariology

Many of the problems encountered in experimental malariology are related to the fact that the technology of maintenance of parasites in the laboratory is poorly developed; culture can be achieved to only a very limited degree. Furthermore, host specificity of malaria parasites is very high, restricting to a great extent the choice of experimental hosts to be used for a given parasite. The organisms pathogenic for man are infectious in only a very limited number of other animals, all of them primates. However, the number of species of malaria parasites is very great, so that a variety of model systems are available for experimental purposes. Although more than 100 species of malaria parasites have been described only the few of greatest usefulness to the experimental immunologist need be considered here. The four parasites traditionally considered human pathogens are: Plasmodium falciparum, the etiologic agent of malignant tertian malaria; P. vivax,

the agent of benign tertian malaria; P. malariae, which causes quartan malaria and P. ovale which is responsible for another form of benign tertian infection, usually called ovale malaria. Maintenance of these organisms in the laboratory involves the use of either chimpanzees, which must be splenectomized prior to infection, or one of several new world monkeys, especially Aotus trivirgatus, the owl monkey.

For many purposes experimentalists have considered nonhuman parasites to be suitable for elucidating many of the general principles of malariology. Among these are several simian malaria parasites which also have been shown to be infectious for man. Prominent among these is P. knowlesi, a natural pathogen of the crab eating macaque, Macaca fascicularis. Whereas this parasite causes a moderately severe disease in its natural host, it is lethal in rhesus monkeys (M. mulatta) within about one week after inoculation with very small numbers of parasites. Much of the usefulness of P. knowlesi as an experimental model for immunological studies is related to this fulminant course and to the fact that it tends to give rise to a synchronous infection; i.e. at any given time, the developmental forms in the blood are similar. P. knowlesi has been known to be infectious for man for many years, and in fact has been used in fever therapy for neurosyphilis. Recently, documentation for a natural human infection with P. knowlesi has been obtained, raising the specter of zoonotic malaria in situations where humans are in proximity to the appropriate simian reservoir hosts and vectors.

P. cynomolgi, a parasite of the rhesus monkey, is useful experimentally since it closely resembles P. vivax of man in both morphology and in the course of the infection it induces in its natural host. This parasite is also infectious for man, as shown in a laboratory accident in which a vivax like disease was induced. On the other hand, P. vivax does not infect rhesus monkeys.

Of very great usefulness in experimental studies is the rodent parasite P. berghei. The natural host of this parasite is the South African tree rat Thamnomys surdaster, but the large majority of studies in the laboratory have been conducted with more easily obtainable laboratory rodents. Most strains are highly virulent for mice; 1×10 parasitized erythrocytes kill the animals in approximately one week. The parasites are also infectious for rats in which the degree of virulence is a strong function of age. Very young rats are killed with moderate doses of malaria parasites, whereas older rats develop only a low grade parasitemia. Rats not yet mature show an immediate degree of susceptibility. This host species thus provides a spectrum of susceptibilities which can be exploited for immunological studies in malaria. A disadvantage of the rat as an experimental host is the limited duration for which experiments can be designed. Variation in counts between individual animals also tends to be high. A third host species which has found some usefulness is the hamster, which is less susceptible than mice but more susceptible than mature rats.

A large number of studies have been conducted with avian malaria parasites. Many of the most productive studies have involved P. gallinaceum of the domestic chicken. The duck parasite P. lophurae and the canary pathogen P. cathemerium have also been investigated extensively. These avian species have advantages for certain aspects of the study of the host response to malaria.

Fundamental to most experimentation in malariology is the maintenance of parasites in the laboratory. Most investigators choose to maintain the parasites by blood passage. In essence, this consists of the injection of the recipient animal with blood from an infected donor. In this way the life cycle of the parasite is in effect short-circuited to consist of the asexual stages only. The details of the procedure vary with the parasite and with the requirements of the investigator. Typically, P. berghei is maintained by a twice weekly passage in mice or weekly passage in rats. Most often, approximately 1×10^6 parasitized erythrocytes are administered intraperitoneally; a somewhat more reproducible course of infection can be induced by intravenous injections. The diluent employed is not crucial as long as the requirement of protection of the host red cell is met. In this laboratory, citrated saline anticoagulant, used for collection of blood, is used also as a vehicle for routine passage; phosphate buffered saline with added mouse or rat serum is used for some experimental work. Refrigeration of parasites during manipulations improves their survival. Quantification of inoculum dose is usually accomplished by (a) an estimation of the percentage of erythrocytes parasitized from thin blood film stained with Giemsa's stain and (b) an erythrocyte count; the concentration of parasitized cells in the preparation is then calculated. In this laboratory, a useful alternative procedure for determining the concentration of Plasmodium berghei inoculum is used; parasitized cells are enumerated directly in a cell counting chamber after vital staining with Nile blue sulfate. The procedure does not work well with P. falciparum, due to the small size of the early trophozoites. It has not been evaluated for other species.

Monitoring the course of malaria infections has been accomplished by a number of methods, most of which involve the use of blood films stained with a Romanowsky stain. When parasitemias are very high, a determination of the percentage of erythrocytes parasitized using conventional thin blood films is very useful. Where quantitation of parasitemia over a greater range of concentrations is desirable, various methods using thick blood films can be employed. The thick blood film, as its name implies, consists of a very heavy film of blood on the microscope slide. The erythrocytes are lysed by exposure to water, leaving only the poorly staining stroma, nonhemoglobin containing elements, and parasites. The concentration of parasites in the blood is determined by reference to the number of white cells in the preparation or by a knowledge of the volume of blood from which the counts are obtained. A method for enumerating P. berghei parasitized erythrocytes in rat blood using an electronic particle counter has also been developed; saponin is used as a hemolytic agent so that the collapsed

parasitized cells (primarily reticulocytes) can be counted electronically. This method has not been worked out for systems in which the predominant cell type is the mature erythrocyte, in which case the residual particles after saponin lysis are much smaller.

When it is necessary or desirable to induce malaria by the more nearly natural sporozoite method, the technology becomes much more complex. Mosquitoes must be maintained in the laboratory and planning for an experiment involves many more preparatory steps. The mammalian host must be monitored for intravascular development of the sexual stages, the gametocytes, necessary for infection in mosquitoes. Subsequently, the mosquitoes must be monitored for development of sporozoites. These methods are well worked out for a number of host-parasite-vector systems.

Serologic techniques in malariology range over a wide variety of procedures. Although precipitin and complement fixation tests were employed many years ago, the thrust of recent serologic studies has employed the indirect fluorescent antibody test. In addition, indirect hemagglutination tests as well as a soluble antigen fluorescent antibody assay have been employed in recent years. Whereas serologic tests have been very useful in the understanding of some epidemiological questions in malariology, they have been of only limited usefulness in studying the functional immunity developed during the course of these diseases. There is no indication that serologic positivity is evidence for a sufficiently intense functional immunity to be of importance as regards the outcome of challenge. It seems likely that serologic analyses will become more important in the future as more information is obtained on the antigens involved and more rigor is introduced in quantitative methods for estimating serum antibody concentrations.

A technique of accelerating usefulness in research in malariology is that of limited culture of the parasites. Although it has proved thus far to be impossible to maintain malaria parasites indefinitely in culture, limited growth can be an extremely useful tool in the study of malaria immunology as well as other areas in which measurement of physiological parameters of the malaria parasite are important. Since malaria parasites usually go through only one replicative cycle in culture, even under the best conditions, the choice of parameters used to assess growth and development is limited. Evidence for development has often been obtained through morphological observations on the change from immature to mature trophozoites and finally to schizonts. Another technique involves determination of the increase in the number of parasites during the culture period. Recently a marked cell technique has been employed for the detection of penetration of new red cells in culture. Fetal erythrocytes serve as the newly infected host cells and are distinguished from the adult erythrocytes which compose the inoculum by the differential elution of hemoglobin A and F in acid. Perhaps the most useful method of assessing physiologic status of malaria parasites in culture is the use of radioactive precursors of parasite materials to monitor biosynthetic capability. For example, amino acids have been used as precursors of proteins and

orotic acid as a precursor of nucleic acid. It seems likely that the use of these techniques will increase over the next few years. The goal of continuous culture, on the other hand, still seems distant.

III. The study of Immunity to malaria in humans

Because of the fact that immunity to malaria is rarely complete, it is difficult to appreciate its existence in casual observations on a hospital population, particularly in areas of relatively low endemicity and therefore low antigenic stimulation. Physicians are likely to observe patients returning from treatment with second, third or more episodes of malaria. However, in areas holoendemic for malaria those who suffer the greatest morbidity and mortality from the disease are the children. Older individuals, although not completely protected from infection, usually experience less severe illness. On the other hand, an individual from a nonendemic area who contracts the infection can experience disease which is just as severe as that in the children in the local populations. This experience has been well documented in a population in the Gambia in terms of a fall in prevalence of falciparum malaria with advancing age. Abundant evidence for immunity to P. vivax, P. ovale and P. malariae is also available.

That much of the naturally acquired immunity to P. falciparum infections in the human population in Africa is due to antibody has been demonstrated convincingly by passive transfer experiments. IgG was isolated from plasma obtained in the Gambia for use in these studies. Control IgG was prepared from plasma obtained in the United Kingdom. These preparations were used in massive doses as an experimental therapeutic measure in children with acute falciparum malaria. Clear evidence of a therapeutic effect was obtained with the globulin obtained locally but not with the control material.

Three kinds of populations of humans with malaria infections, (namely hospital populations, residential populations and volunteer populations) offer abundant opportunities for studies into the immunology of malaria to the investigator with access to them.

IV. The study of immunity to malaria in laboratory animals

Much of the information presently available on the mechanisms involved in immunity to malaria has been derived from the study of malaria in laboratory animals. In the discussion to follow, a number of malaria parasites and host animals will be considered. Although species differences obviously occur, the main features of immunity to malaria have appeared in common among most of the models studied; it, therefore, seems reasonable to expect that many of the features elucidated with one host-parasite system will apply to many others, hopefully including human infections.

In the 1930's it was already clear that the spleen, liver, and bone marrow exhibited a marked phagocytic response during malaria infection.

This response is characterized by an increase in size and activity of the macrophages with an accumulation of parasitized cells, intracellular destruction of the parasites, and the storage of the resulting malaria pigment, a phenomenon which is readily observed grossly by changes in color of these organs. The other changes in the spleen are also easily observed grossly in terms of the massive splenomegaly which often accompanies acute malaria. These changes, which occur relatively slowly during a primary attack of malaria, occur more rapidly on subsequent exposure, and therefore demonstrate a prime characteristic of the specific immune response, immunologic memory. More recent data indicate that the macrophages thus activated not only phagocytize P. berghei parasitized erythrocytes but that there is phagocytosis of normal erythrocytes as well. It has also been shown that macrophages from the peritoneal cavity of mice infected with BCG phagocytize erythrocytes parasitized by P. knowlesi much more avidly than macrophages from uninfected animals. Thus, such hyperactive phagocytes are produced both by malaria infection itself and by BCG. It is unclear as to whether or not the mechanism is similar in the two instances. The accumulated evidence is great that phagocyte activation by BCG is via the cellular immune mechanism elucidated by Mackaness in which delayed hypersensitivity is a constant correlate. However, delayed hypersensitivity is a constant correlate. However, delayed hypersensitivity reactions have not been demonstrated in malaria infections, although they probably occur in animals immunized with malaria antigen in complete Freund's adjuvant. Malaria also differs from other infectious diseases in which cellular immune mechanisms have been demonstrated in that the mammalian malaria parasites do not parasitize macrophages.

If macrophage activation in malaria is mediated through factors released by immune lymphocytes, passive transfer of immunity with lymphocytes from immune animals should be possible. This has in fact been demonstrated in rats infected with P. berghei, but it is not clear whether the lymphocytes act by virtue of release of mediators or by antibody synthesis. There is in fact no reason to believe that the latter situation is not the case. An experiment which bears on this question was performed by Lourie, who found that antilymphocyte serum (ALS) inhibited the ability of rats to spontaneously clear P. berghei parasites (i.e. to exhibit an active immune response), but that this ALS inhibition could be reversed by the coincident administration of antibody with protective activity.

The roles of thymus dependent (T) and independent (B) lymphocytes in immunity to malaria are under investigation. It has been demonstrated that neonatal thymectomy enhances P. berghei infections in rats, but that self cure, though delayed, still occurs. On the other hand, embryonic bursectomy severely impedes the development of active immunity and the expression of passive immunity to P. gallinaceum infections.

The effect of splenectomy on latent malaria has been documented on many occasions over the years. Immunity to some malaria parasites

appears to involve an equilibrium between parasite replication and host defense, so that latent infection persists and when splenectomy is effected, parasites often appear in the peripheral blood. Splenectomy is consequently used as a device for enhancement of susceptibility to malaria. In addition to the sequestration of both parasitized and nonparasitized erythrocytes, the spleen is believed to be able to remove parasites from erythrocytes, returning the deparasitized erythrocytes to the peripheral blood. It seems likely that splenic sequestration is a function of both enhanced phagocytic capability and abnormalities of the erythrocyte membrane. Membrane alterations occur in both parasitized and nonparasitized erythrocytes during malarial infections. Although quantitative comparison with other sites has not thus far been experimentally feasible it seems reasonable to consider that the spleen is one of the primary sites for the removal of parasites from the circulation.

In addition to induction of immunity by virtue of infection and recovery, animals can be immunized by the administration of nonreplicating parasites and extracts thereof. Some success with artificial immunization was obtained in the 1940's: for example, Jules Freund who induced immunity to P. knowlesi by the injection of killed parasitized cells in the adjuvant which bears his name. It has been possible on numerous occasions since that time to induce immunity through the use of Freund's adjuvant. However, in the absence of adjuvant, most attempts to immunize have been unsuccessful or less effective than infection and cure. Some success with extracts of P. berghei has been obtained in rats by Zuckerman. A further advance has been the achievement of protection using partially purified antigens. Success has also been reported using similar partially purified antigens in mice.

Immunization through the use of irradiated parasites has proven to be an effective experimental procedure. Both mice and rats can be immunized with irradiated P. berghei parasitized erythrocytes, and Aotus monkeys can be similarly immunized with P. falciparum parasitized erythrocytes which have been exposed to 20-25 kr of gamma irradiation from a Co-60 source. Attempts to recover immunogenic fractions from these irradiated parasitized cells have thus far failed. In fact, lysis of the parasitized cells abolishes immunogenicity. These results raise the question as to whether or not metabolically active parasites are required for immunogenicity; it is possible that the effective immunogen is a product of the irradiated parasite rather than a preformed moiety in the inoculum. However, the immunogenic activity of irradiated parasites is not blocked by the simultaneous administration of sulphadizine, an agent which is highly effective against P. berghei and which might be expected to inhibit biosynthesis of antigen in vivo.

Irradiated sporozoites of P. berghei have also proven to be highly effective immunogens. In this system as well, disruption of the parasites inhibits immunogenicity. The immunity to disease obtained by irradiated sporozoite immunization is "all or none" in the sense that unless erythrocytic invasion is completely prevented, the pathological

consequences of the infection, although possibly delayed, are ultimately the same as in nonimmune animals. There is no apparent cross protection between erythrocytic and exoerythrocytic stages; animals immunized with sporozoites have no immunity to blood induced infection; animals immune to blood forms show immunity to the development of erythrocytic forms after infection with either sporozoites or blood forms. The development of exoerythrocytic stages in animals immunized with blood forms has not been studied.

Studies on the expression of immunity have made it quite clear that antibody is a determinant of the outcome of malaria infection in many host parasite systems. It was demonstrated many years ago that serum from immune animals is effective in the modification of P. knowlesi infections in rhesus monkeys and a decade ago that P. falciparum infections in humans can be similarly influenced. Studies have also been performed demonstrating a crucial role for antibody for the outcome of infections due to P. berghei in the rat. Antibody is effective in delaying the course of mouse infections, although death is not prevented.

The effect of antibody on malaria parasites can be studied by an in vitro assay for antibody directed against P. knowlesi described by Cohen et al. In this system the incorporation of radioactive labeled amino acid into parasite protein is used as a device to estimate the physiologic status of the parasite. In the presence of antibody, protein synthesis is essentially normal until the time of schizogony at which time further incorporation is inhibited in the presence of an excess of antibody. This information strongly suggests that the site of action of the antibody is on either the schizont or the extracellular merozoite. Most available data are in concert with this concept; antibody which is highly effective against P. berghei in vivo causes no agglutination of parasitized erythrocytes. Furthermore, in experiments designed to test the permeability of the red cell membrane, antibody and complement induced no damage. In the in vivo studies of Cohen et al. no immediate effect on parasitemia was observed by the administration of immune serum; it was only at the time of schizogony that the difference between patients treated with the immune serum and the controls emerge.

Some additional information is available relevant to the identity of the target cell(s) of protective antibody in malaria. It has been known for many years that the schizonts of P. knowlesi can be agglutinated in the presence of antibody; i.e., by some mechanisms, antigen is available on the surface of these cells. Recently, Brown has demonstrated that in addition to the agglutinin, a schizont opsonizing antibody can also be demonstrated in the serum of monkeys immune to P. knowlesi. Data on other species of malaria parasites with respect to such antibodies are sparse and the possibility that P. knowlesi is peculiar in this regard cannot be dismissed. A more likely explanation for the paucity of data on agglutination of erythrocytes infected by other parasites, however, is the fact that the collection of large numbers of schizonts is expedited in P. knowlesi infections due to

(a) the ability of the parasite to induce high parasitemias and (b) its synchrony. At this point in time it seems likely that the schizont and the merozoite might both be attacked by antibody.

The question of the mode of action of the antibody on the target cell has been approached in a number of ways. By analogy with other systems, it can be speculated that the antibody sensitizes the target cell in the presence of complement components for cytotoxic reaction and/or phagocytosis. Thus, the effect of complement on antiparasitic activities is an important object of study. A number of workers have demonstrated a reduction in the level of complement components during malaria due to P. berghei, P. knowlesi and P. gallinaceum. However, in the in vitro system described above, F(Ab')₂ is as effective as is intact IgG. Furthermore, heat treatment of all of the serum additives in the culture system does not influence the antiparasitic effect. Recent studies have demonstrated that the cobra venom factor which destroys the third component of complement does not influence the protective effect of passively administered antibody in P. berghei infections of the rat. The evidence, to date, therefore, suggest that complement is not required for the antiparasitic effect of antibody. Destruction of the target cell by either a cytotoxic reaction, by phagocytosis and intracellular destruction, or by inhibition of penetration of a new host cell (i.e., neutralization) could all conceivably proceed in the absence of complement components. At this point in time the latter mechanism is favored by the evidence.

V. The antigenic structure of malaria parasites

Not unexpectedly, malaria parasites have proved to be enormously complex antigenically. This is perhaps best exemplified in the studies of Wilson et al. who have demonstrated not only a large number of antigens within a given extract of P. falciparum from a single patient but an almost bewildering array of different antigens and antibodies in different individual patients. There is broad cross reactivity between the parasites of man and monkeys as shown by fluorescent antibody studies, but very little between these and parasites of rodents or birds. An exception is the reaction of serum from patients with P. falciparum to a constituent of P. gallinaceum. These serological relationships have no relevance to functional immunity to malaria parasites; studies in human volunteers have shown both species and strain specificity on cross challenge of semi-immune volunteers. Species specificity appears to extend to effects passively transferrable by antibody. East African gammaglobulin is effective against West African P. falciparum malaria, and an inhibitory effect on P. falciparum of Asian origin by African IgG can be detected in the Aotus monkey system.

In addition to the features of strain and species specificity, malaria parasites apparently have the ability to modulate their antigenic specificity in response to immune pressures. Thus, it has been shown by the schizont agglutination test that P. knowlesi parasites which persist in rhesus monkeys after the development of agglutinating anti-

body is no longer reactive with the antibody; i.e., parasites or multiple specificities can evolve sequentially in a single animal. This situation also obtains for protective antibodies as has been shown for P. berghei. In spite of this very serious mechanism of interference with host immune processes, protective immunity is established in many host-parasite systems. Immunity has been induced in both systems where antigenic variation has been studied in most detail (i.e., P. knowlesi in rhesus monkeys and P. berghei in mice). It is clear that in these situations the failure of host defense through antigenic variation can be circumvented. Brown has suggested that the primary exposure to malarial antigens endows the host with immunologic memory so that on subsequent exposures to a different variant a secondary immune response occurs. In this model the variant specificity would be analogous to a hapten which would function with a carrier to which the host was already immune.

2. Cryopreservation of erythrocytes parasitized with Plasmodium falciparum.

A method developed for freezing and storing human erythrocytes for transfusions has been applied to storing of Plasmodium falciparum parasitized erythrocytes obtained from infected owl monkeys (Aotus trivirgatus). The method involves using a cryoprotective agent containing glycerol and reconstituting isotonicity stepwise after thawing the cells and before they are used for infecting laboratory animals or for in vitro experiments. The cells retain both the capacity to incorporate C-14 isoleucine into trichloroacetic acid precipitable material after storage (protein synthesis) and to infect animals. Experiments of limited duration (6-8 hours) indicate that the rate of protein synthesis by cells which have been stored in the frozen state approaches that observed with fresh preparations. Studies on the further in vitro development of stored malaria parasites and on the effect of prolonged storage are in progress. Retention of infectivity to Aotus monkeys is superior to previously used methods as judged by the short pre-patent period. Studies on the persistence of infectivity are also being performed. Changes in the capacity to synthesize protein and to infect animals have not been detected during serial experiments over a seven week period.

3. Immunosuppression of the protective response to Plasmodium berghei in mice.

In attempts to better understand mechanisms of the immune response drug induced antigen specific immunosuppression has been widely used. Cyclophosphamide was used in attempts to selectively suppress the immune response of adult mice to Plasmodium berghei by giving the drug along with malarial antigen. Protective immunization was later attempted by injecting potentially protective antigen. During the final phase, the mice were challenged with the parasite and the degree of immune responsiveness to immunization, as a function of pretreatment manipulation, was evaluated. This was accomplished by studying post-challenge

mortality for a 30-day period. The extent of possible drug related nonspecific immunosuppression was examined by analysis of sheep erythrocyte hemolysin activity in mice challenged with sheep erythrocytes immediately following pretreatment manipulation. At the time these mice were challenged with sheep red cells, the remainder of the mice were being immunized prior to subsequent malaria infection.

The immune response of the mice to the parasite is markedly suppressed by previous exposure to either the drug alone or the drug given with malaria antigen. In the latter case, there is an acceleration in death rate late in the post-challenge period as well as a higher total mortality 30 days after challenge than when animals are treated with the drug alone. Confirmatory work has shown mortality data to be highly reproducible. Chemosuppressive phenomena were not a grossly nonspecific type as shown by continued production of sheep red cell hemolysin in all animals regardless of mode of pretreatment. These observations suggest the induction of partial tolerance to the antigens involved.

Task 00 Malaria Investigations

Work Unit 129 Host responses to malaria

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION ^a	2. DATE OF SUMMARY ^a	REPORT CONTROL SYMBOL	
				DA OA 6525	73 07 01	DD-DR&E(AR)636	
3. DATE PREV SUMRY ^a	4. KIND OF SUMMARY	5. SUMMARY SCTY ^a	6. WORK SECURITY ^a	7. REGRADING ^a	8. DR&E INSTR ^a	9. SPECIFIC DATA CONTRACTOR ACCESS ^a	10. LEVEL OF SUM ^a
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11. NO./CODES ^a	PROGRAM ELEMENT		PROJECT NUMBER		TASK AREA NUMBER	WORK UNIT NUMBER	
	63713A		3A663713D829		00	132	
a. PRIMARY							
b. CONTRIBUTING							
c. RESEARCH		CDOG 114(f)					
11. TITLE (Precede with Security Classification Code) ^a							
(U) Clinical and Metabolic Studies of Malaria							
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00 2600 BIOLOGY							
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b. NUMBER ^a				FISCAL		73	
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				74		2	
						95	
19. RESPONSIBLE DOD ORGANIZATION				20. PERFORMING ORGANIZATION			
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21. GENERAL USE				ASSOCIATE INVESTIGATORS			
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				NAME:			
				DA			
22. KEYWORDS (Precede EACH with Security Classification Code)							
(U) Malaria; (U) Antimalarials; (U) Parasite; (U) Red Blood Cell							
23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRAM (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
<p>23. (U) Study pathophysiology of acute falciparum and vivax malaria, and assess various modes of antimalarial therapy with respect to clinical responses and radical cure. Document metabolic alterations of human and animal red blood cells when infected with malaria parasites and assess the effect of antimalarial drugs on these alterations in order to develop new drugs effective against resistant falciparum malaria.</p> <p>24. (U) Document clinical features of acute disease, evaluate available therapeutic agents with respect to clinical response and radical cure; provide surveillance for toxicity and efficacy testing of new antimalarial agents by government contractors; provide expert consultation on treatment of resistant falciparum infections and secure new strains of malaria for introduction into the volunteer test program. Measure the effects of antimalarial drugs on morphologic growth and radiolabelled precursor incorporation into protein and nucleic acids during in vitro schizogony. Measure folic acid reductase in parasite suspensions.</p> <p>25. (U) 72 07 - 73 06 Consultations with many physicians treating patients for malaria from Vietnam continued on an informal basis. Several new drugs and new drug combinations were introduced into ten clinical test centers, and cooperative field trials with investigational compounds in Thailand were initiated. In vitro culture of P. knowlesi parasites has continued as a screening procedure for antimalarial activity and for demonstration of drug potentiation. Analogs of purine bases and nucleosides which were effective during screening have been studied for establishment of relative effectiveness. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 72 - 30 Jun 73.</p>							

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PII Redacted

Project 3A663713D829 MALARIA PROPHYLAXIS

Task 00 Malaria Investigations

Work Unit 132 Clinical and metabolic studies of malaria

Investigators.

Principal: COL Craig J. Canfield, MC

Associate: Gerald J. McCormick, Ph.D., Esther P. Jorolan, Ph.D.,
Gloria P. Willett

Description.

The objectives of this work unit are to assess the clinical state and therapeutic response of patients to acute falciparum and vivax malaria, provide surveillance for toxicity and efficacy testing of new antimalarial agents by contractors, provide expert consultation on treatment of resistant falciparum infections, secure new strains of malaria for introduction into the volunteer test program, study metabolic pathways of the host red blood cell parasite complex and to assess the effect of antimalarial drugs on these pathways in order to develop new drugs effective against resistant falciparum malaria.

Progress.

Data on the number of cases of malaria in Vietnam during 1965-1971 was compiled and published (1). The rarity of resistant falciparum malaria in Negro soldiers in Vietnam was documented and published (2).

Admission to Walter Reed General Hospital for acute or recrudescent falciparum malaria virtually ceased during the reporting period. An undocumented number of telephone consultations were provided on problems associated with malaria infection from a variety of civilian and military treatment facilities throughout the United States.

The principal investigator of this work unit also served as principal investigator for all new antimalarial drugs undergoing evaluation in the clinical centers and in field studies in Thailand. Four new IND's and 14 annual supplements were submitted to the Army Investigational Review Board. All volunteer and field studies with 2 of these drugs, WR 33063 and WR 30090 were compiled and published during the reporting period (3). Both drugs were found to be better than any single drug presently available for treatment of resistant falciparum malaria and were virtually free from adverse effects. Coordinates of clinical studies of even more active analogs continue.

One new strain of falciparum malaria was introduced into the clinical center. This strain was obtained by Dr. Clyde in Sabah and was found to be both chloroquine and pyrimethamine resistant.

In vitro study of synergism between antimalarial drugs has continued, using the ^{14}C -orotic acid system. The studies which were presented at the Malaria Workshop at WRAIR were published (4). Combinations of pyrimethamine and folinic acid were found to be antagonistic in this system.

Dose response studies of analogs of purine bases and nucleosides have been done to establish relative effectiveness of those which were effective in the drug screening procedure. The most effective compounds were puromycin and tubercidin relative to both RNA and DNA, and 3'-amino-3'-deoxy-adenosine relative to RNA only. Cordycepin (3'-deoxy-adenosine) was not effective in these experiments.

Results from the collaborative study with Dr. de Zeeuw which he presented at the Malaria Workshop were published (5). Analysis of lipid components was transferred from the Netherlands to the University of Maryland.

Project 3A663713D829 MALARIA PROPHYLAXIS

Task 00 Malaria Investigations

Work Unit 132 Clinical and metabolic studies of malaria

Literature Cited.

Publications:

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL	
				DA OB 6495	73 07 01	DD-DR&E(AR)636	
3. DATE PREVIOUS	4. KIND OF SUMMARY	5. SUMMARY	6. WORK SECURITY	7. REGRADING	8. DISEM INSTR	9. SPECIFIC DATA CONTRACTOR ACCESS	10. LEVEL OF SUMMARY
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10. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER		WORK UNIT NUMBER		
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b. CONTRIBUTING							
c. CONTRIBUTING	CDOG 114 (F)						
11. TITLE (Precede with Security Classification Code)							
(U) Malaria Investigations (09)							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS							
012100 Organic Chemistry							
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
73 07		CONT		DA		C. In-House	
17. CONTRACT/GRANT				18. RESOURCES ESTIMATE		19. PROFESSIONAL MAN YRS	
a. DATES/EFFECTIVE: NA				PRECEDING		b. FUNDS (in thousands)	
b. NUMBER				FISCAL YEAR		c. FUNDS (in thousands)	
c. TYPE				73		9	
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e. F.C.U.M. AMT.				74		16.1	
f. F.C.U.M. AMT.						620	
20. RESPONSIBLE DOD ORGANIZATION				21. PERFORMING ORGANIZATION			
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23. KEYWORDS (Precede EACH with Security Classification Code) (U) Malaria; (U) Drug Development; (U) Antimalarials; (U) Biology; (U) Chemistry; (U) Pharmacodynamics; (U) Drug Metabolism; (U) Toxicology							
24. TECHNICAL OBJECTIVE, 25. APPROACH, 26. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
<p>23. (U) To manage, to integrate, and to provide technical direction for the contract Drug Development Program on Malaria. To conduct in-house studies in pharmacology, biology and organic synthesis specifically related to the design, development and exploitation of new antimalarials for military use.</p> <p>24. (U) Close supervision of the Malaria Program will be maintained by defining necessary research areas, by providing guidance and an integrated evaluation of productivity, and by the redirection and coordination of objectives as dictated by feedback from clinical studies of candidate antimalarials. The development and exploitation of more effective chemical structures and of animal models for the study of toxicity, drug metabolism and pharmacodynamics of new antimalarials will continue in support of IND requirements for clinical drug trials, and to provide a basis of predicting soldier response to these agents under the stresses of a military environment.</p> <p>25. (U) 72 07-73 06 Progress was previously reported under 3A663713D829 as work units 114,171 and 122. Technical management continued for 50 contracts in chemistry, 9 in biology and 11 in pharmacology. Four new IND applications and 15 supplements were submitted and approved. Cardiovascular and pulmonary pharmacology was investigated in dogs, and drug metabolism in mice, for 4 new drugs. Hepatic microsomal modifications of 9 drugs in mice, and the hemolytic effects in vitro of 6 drugs were studied. For technical reports see Walter Reed Army Institute of Research Annual Progress Report. 1 Jul 72-30 Jun 73.</p>							

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Project 3A663713D829 MALARIA PROPHYLAXIS

Task 00 Malaria Investigations

Work Unit 134 Malaria Investigations

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General

This program of malaria investigations is a modern medicinal chemistry research effort designed to develop drugs to treat and/or prevent P. falciparum and P. vivax infections of the soldier. The fields of investigation logically include synthetic organic chemistry, biology and pharmacology. Close supervision is maintained by defining necessary research areas, by providing guidance and an integrated evaluation of productivity, and by the redirection and coordination of objectives as dictated by feedback from clinical studies of candidate antimalarials. The development and exploitation of more effective chemical structures and of animal models for the study of toxicity, drug metabolism and pharmacodynamics of new antimalarials supports the Investigational New Drug requirements for clinical drug trials, and provides a basis of predicting soldier response to these agents under the stresses of a military environment. Program objectives are being attained through the combined efforts of a team of research managers with the necessary expertise, in-house laboratory support in the areas of synthetic chemistry, biology and pharmacology, and extramural consultation.

The Contract Chemical Synthesis Program

At the end of FY-73 there were 33 active contracts to be carried over into FY-74; 12 contracts were terminated during the year.

In addition to the research synthesis contracts there have been four active preparations laboratories to resynthesize, on a larger scale, selected compounds that are needed for large animal testing, toxicological studies and clinical investigations. There has also been one contract to synthesize radioactively tagged compounds and one to analyze and confirm the purity and identity of compounds and compositions to be used in preclinical and clinical studies.

During FY-73 there was a total of 1,000 compounds submitted by the synthesis contract program, 700 of which were target compounds synthesized on a rational basis. The average cost per target compound, calculated from the inception of the program, is slightly less than \$2,400. The preparations laboratories continued to work at full capacity. The number of compounds requested from these laboratories during the year was 42, 3 and 4 in small, medium and large quantities respectively; compounds received numbered 46, 1 and 1 in these categories respectively. Publications and manuscripts generated by the synthesis contractors during the year totaled 21; 3 patents were issued.

In the synthesis program, other than a few pyridine- and phenanthrene-methanols with 3 and 4 carbon side chains, the naphthalene methanols and the anthracene methanols were the only classes of the aryl amino alcohols in which additional work was indicated, and which were carried over from last year. These now are being phased out. The naphthalene methanols seem to be at least as active as their nitrogen analogs in the primary screen and selected ones will be carried to advanced screening. The anthracene methanols did not appear to be quite as active as the phenanthrenemethanols but such activity probably could be developed with additional work. However, the inherent synthetic difficulties militated against the possibility of the emergence of a practical drug in this class.

The greatest emphasis during the past year was placed upon the synthesis of prophylactic and curative agents. The primary thrust was in the areas of the 6- and 8-aminoquinolines. Other areas of potential prophylactic activity now under study include the aminonaphthyridines, the 2-aryl-2-piperidyltetrahydrofurans and derivatives, and Endochin-related compounds. Both the synthesis and the screening of the 6- and 8-aminoquinolines proved to be difficult. However, testing in primates has shown that certain of the new 8-aminoquinolines may compare favorably with primaquin.

A second broad area of endeavor encompassed the synthesis of compounds that are loosely, and somewhat arbitrarily designated as metabolic inhibitors. These include, for example, folic and amino acid inhibitors. Thus there are being synthesized modifications of folic acid itself as well as appropriately substituted pyrrolopyrimidines, quinazolines, pyrimidines, aza- and deaza-quinazolines, and purines, including nucleosides. The most active compounds uncovered, in terms of milligrams per kilogram, are in the 6-arythio-2,4-diamino-quinazoline class.

Synthesis of the phenolic Mannich bases, the bis amidines and the cinnamoyloxazolinyl piperazines, all of which were based upon active prototype compounds, has been continued. New synthesis programs have been started to exploit the activity uncovered in the aryl-alkanesulfonates and in the antibiotics Clindamycin and Threomycin.

The synthesis program designed to exploit the lead uncovered in the activity of gem dithiols and polysulfides, while developing more active compounds than the prototypes, did not yield compounds of the caliber desired and was dropped. Also phased out was the work on the 6-hydroxy-7-alkylthioquinoline-quinones because of disappointing activity in the primate test systems. Finally, the synthesis of the cycloalkyl peroxides was phased out because of the inherent instability of the compounds.

Acquisition of Compounds

In addition to the 1,100 compounds received from the synthesis program, 11,600 were acquired under the Cooperative Industry-Government Agreement for the Testing of Compounds, and 1,400 more were received as gifts. The WRAIR bottling team made eight sample collection visits collecting 6,400 compounds.

Renewed emphasis has been put on reviving the collaboration of companies that have been inactive for some time; three inactive firms chose to terminate the Agreement. Sixteen new companies were contacted, seven of which signed the Agreement. Competition for chemicals has increased markedly during the year with the letting by the National Cancer Institute of a large contract solely for the purpose of contacting potential submitters and acquiring new chemicals; in addition the NCI has established a new office in Europe for the same purpose.

The Organic Laboratory Synthesis Program

The in-house synthesis program has as its objective the conversion of some of the highly active quinoline- and phenanthrene- methanols into their sulfur analogs, both in the form of the free thiol and as a physiologically potential thiol. The conversion of quinine resulted in the compound 9-deoxyepiquinine-9-thiosulfuric acid which had the same order of activity as quinine. Heretofore, modification of the hydroxyl in the aminoalcohols, with the exception of the conversion to the oxazolidine, resulted in loss of activity.

Biology

All compounds received by the Division of Medicinal Chemistry, WRAIR are evaluated for anti-malarial activity in a blood-induced P. berghei mouse screen and/or a sporozoite-induced mouse (P. berghei yoeli) or chick (P. gallinaceum) screen. Active compounds are further evaluated for other characteristics as described below.

a. Primary Screens

1. All primary testing of compounds for blood schizonticidal activity is conducted by Dr. Leo Rane at the University of Miami. The endpoint of the system is extension of survival time of ICR/HA Swiss mice that are given a standard inoculum of P. berghei (KB6173) on day zero and a single subcutaneous injection of the test agent in peanut oil on day 3, i.e. 72 hours later. Untreated animals die within 6 to 8 days. An increase of 100% in mean survival time is considered the minimum effective response for a candidate compound. Animals that survive 60 days are scored as "cures". During this report period 14,276 compounds were tested, 709 were found to be active.

2. Primary testing of compounds for prophylactic activity has been conducted using a sporozoite-induced P. gallinaceum infection in white Leghorn cockerels and a sporozoite-induced P. berghei yoeli infection in HA/ICR mice. The chick test is a high volume screen. The mouse test has a much lower throughput rate. The latter is being discontinued because (a) of the high efficiency of the chick screen, (b) the difficulty in maintaining a viable colony of Anopheles mosquitoes required for the sporozoites for infection, thus, resulting in the test being inoperable much of the contract period, and (c) the cost of operating two primary prophylactic screens. During the current report period 2,624 compounds were tested in the avian system. Three hundred eighty-seven were found to be active. In the rodent system 304 compounds were screened. Of these 117 were shown to have prophylactic activity.

b. Secondary Tests

1. Blood Schizonticidal Systems

a. Selected compounds are evaluated by Dr. Paul Thompson, University of Georgia. The system employs blood induced P. berghei infections in mice to ascertain activity against drug resistant parasites, absorption by various routes of administration, therapeutic index estimation, duration of action and PABA antagonism. During this report period 63 compounds were tested orally against the parent sensitive (P) line and one or more drug resistant lines. Of these, 51 showed activity equal to or greater than quinine. Several combination studies were conducted. Synergism was found when sulfadiazine was administered orally with the quinazoline WR 158,122, the tetrahydrofuran WR 179,305 and the phenanthrene methanol WR 122,455. Synergism was also found when quinine and minocycline (WR 8,778) were administered concurrently.

b. Certain compounds are sent to Dr. Wallace Peters, Liverpool School of Tropical Medicine for evaluation. Included in his work are combination studies. He has found a high level of potentiation between chloroquine and erythromycin when used against infections of moderately resistant (NS) and highly resistant (RC) strains of P. berghei in mice.

c. An in vitro test is conducted by Dr. Karl Nieckmann, Rush-St. Luke's Hospital, Chicago, Illinois. The test consists of incubating human blood parasitized with P. falciparum with test compounds for 24 hours at 38-40°C. After incubation, blood smears are prepared and inspected under the microscope. The extent of the inhibition of the parasite maturation can be ascertained. Four strains of parasites are used - the chloroquine sensitive Uganda strain and the resistant Malayan (Camp), Vietnam (Marks) and Cambodian strains. A particularly gratifying development in monitoring this test system is the finding that there is a very high correlation between the activity of compounds in this simple in vitro test and the activity of the compounds in vivo. During the present report period a total of 71 compounds were tested. Sixty-five were found active.

d. Approximately 20 compounds per year are tested against blood induced simian malaria (P. cynomolgi) in Rhesus monkeys. This testing, conducted by the SEATO Laboratory, Bangkok, Thailand, is done by administering compounds orally at 5 different dose levels for seven consecutive days beginning on the fourth day after infecting the animals. The course of the parasitemia in each animal is determined daily for up to 60 days. The testing is proceeding at a highly efficient level.

e. Further testing is done using the owl monkey, Aotus Trivargatus. Since Dr. Quintin Geiman was first able to establish Falciparum malaria in this small South American monkey several strains have been successfully adapted. To date eight strains of Falciparum and two strains of Vivax have been established in these monkeys by Dr. Leon Schmidt, Southern Research Institute, Birmingham, Alabama. All infections respond to drugs in much the same manner as the corresponding infections in man. Approximately 6 to 8 compounds per year are tested in this system. Of particular interest are studies with the quinazoline WR 158,122 (2,4-diamino-6-(2-naphthyl)-sulfonylquinazoline) in combination with sulfadiazine. Sulfadiazine given alone at a dose rate of 80 mg/kg/day for seven days effected no more than a transitory depression of parasitemia. However, given at 5 mg/kg/day in combination with WR 158,122 the activity of this quinazoline against various strains was enhanced by 16 to 64 fold. Furthermore, no quinazoline resistance was developed and there was no enhancement of the toxicity of the quinazoline when sulfadiazine was concurrently delivered.

2. Prophylactic and Radical Curative Systems

a. More definitive determination of the prophylactic activity of candidate compounds are ascertained by Dr. Harry Most, New York University. Employing 3-4 week old female rats the effects of test compounds on exoerythrocytic (EE) forms of sporozoite-induced P. berghei infections are determined by examining microscopically stained tissue sections prepared from livers of animals given sporozoites 40-45 hours earlier. To make sure no EE forms are overlooked in the tissue sections other rats are inspected daily for parasitemia and if negative for 5 days mice are subinoculated and the parasitemia followed for 6 days. During this report period this test system has been modified to at least double the compound throughput from the previous 6 to 8 compounds per year. During the present reporting period 17 compounds were tested. Five of which had confirmed prophylactic activity. Furthermore, more information at different dose levels is now being received.

b. To determine the extent that a compound is acting as a prophylactic agent as opposed to acting as a residual blood schizonticidal agent, compounds are sent to Dr. Wallace Peters, Liverpool School of Tropical Medicine. Of significance is the fact that the tetrahydro furan derivatives show marginal prophylactic activity.

c. Simian testing for prophylactic and radical curative activity is conducted by Dr. Leon Schmidt. The test employs rhesus monkeys that receive infections of sporozoites of P. cynomolgi. Compounds are administered orally at 1/2, 1/4, and 1/32 the minimum lethal dose for nine consecutive days beginning the day before infection. Examination for parasitemia is begun on the last day of administration and repeated daily until patency or 60 days if negative. This phase of the testing is designed to determine prophylactic properties of a candidate compound. Radical curative properties can be subsequently determined. During this report period preliminary indications are that there are several new 8-aminoquinolines already in the WRAIR inventory or being prepared that are at least as effective as primaquine.

3. Antifol Testing

During the reporting period 87 compounds were evaluated by Dr. C. C. Smith, University of Cincinnati for folic acid antagonism in Streptococcus faecium, Lactobacillus casei and Pediococcus cerevisiae. Of those compounds tested, 77 were active and 10 inactive. Twenty were reversed by addition of folate.

Pharmacology

1. Absorption, distribution and excretion in mice.

The absorption, distribution and excretion of four new anti-malarials after oral administration using radioactive compounds were studied in mice. These were investigated prior to taking the drugs into man for extrapolation to potential human response.

a. WR 171,669-¹⁴C:

At 20 mg/kg the drug appeared to be rapidly absorbed. Plasma levels were relatively constant during the first 6 hours, peaking at approximately 1 µg/ml, and dropping considerably after 24 and 48 hours. Red cell levels were much lower during this period. Tissue localization (excluding the gastrointestinal tract) of the radiolabel after 2 and 4 hours was mainly in the liver, lungs, spleen, kidney, gall bladder and heart. The main route of excretion was via the feces with very small amounts being excreted in the urine and no radiolabel detected in expired air. Thin layer chromatography of feces extracts showed two major and at least one minor peak. One major peak was parent drug, the other major peak was a glucuronide of a metabolite.

b. WR 142,490-¹⁴C:

At 10 mg/kg the drug appeared to be well absorbed. Red cell levels were relatively constant during the first 12 hours, peaking at approximately 2 µg/ml and dropping slowly for days. Plasma levels peaked at 1.4 µg/ml and also declined slowly. Tissue localization (excluding the gastrointestinal tract) of the radiolabel at 24 and 48 hours was mainly in the lungs, gall bladder, kidney, spleen and liver. The main route of excretion was slowly via the feces with up to 20% in the urine and no detectable radioactivity in expired air. The drug was readily metabolized with at least two major metabolites in the feces.

c. WR-143,946-¹⁴C:

At 20 mg/kg the drug was erratically absorbed. Plasma levels were relatively constant during the first 6 hours, peaking at about 3.5 µg/ml and then falling rapidly. Red cell levels were much lower during the entire period. The main excretory route was fecal, with traces in urine and none detected in expired air. Thin layer chromatography of feces extracts indicated very little metabolism of the drug.

d. WR 159,412-¹⁴C:

At 40 mg/kg the drug appeared to be moderately well absorbed. Both plasma and red cell levels peaked at 2-3 µg/ml several hours postdose and then gradually declined. The main route of excretion was via the feces with up to 10% in the urine. No radioactivity was detected in expired air. Radioactivity extracted from feces was primarily parent drug with several minor metabolites while urine contained primarily metabolites.

2. Cardiovascular and pulmonary pharmacology in dogs.

The general cardiovascular and pulmonary pharmacology of four new antimalarials was studied in anesthetized dogs after intravenous, intraduodenal or oral administration. These studies are being used to predict potential effects in humans. In each in vivo experiment, systolic and diastolic blood pressure, heart rate, EKG, and pulmonary rate and depth were monitored and recorded. Dose response curves to epinephrine, nor-epinephrine, isoproterenol and angiotensin were obtained before and after drug administration.

a. WR 171,669:

This candidate antimalarial drug was administered intraduodenally to six dogs in doses ranging from 20 to 100 mg/kg. In addition to the parameters mentioned above, the ascending aortic blood flow was measured by an electromagnetic flow probe and the myocardial contractile force was determined by means of a Walton-Brodie strain gauge arch sutured to the right ventricle in two dogs. To further characterize direct cardiac effects the drug was tested in the isolated, perfused dog heart. WR 171,669 produced no observable change in arterial blood pressure, heart rate, EKG pattern or respiratory rate or depth during a 3 hour observation period. Likewise, cardiac contractile force and ascending aortic blood flow were not affected. Dose response curves before and 1 hour after drug administration were not different.

b. WR 148,946:

Due to the poor water solubility of this chemical, it was prepared as a suspension and administered intraduodenally in doses of 10, 20 and 40 mg/kg of body weight. No significant effects on blood pressure, heart rate or respiratory rate were observed over a 3 hour period. The failure to evoke significant responses directly on the cardiovascular or respiratory systems after intraduodenal administration may be interpreted as evidence that relatively large oral doses could be tolerated with minimal adverse effects on these systems.

c. WR 159,412:

After intraduodenal, i.v. or oral (in capsules) administration of WR-159,412 to eight dogs, in doses ranging from 6 to 320 mg/kg, no significant effects were observed on any of the parameters being monitored, thus suggesting a relatively low level of acute toxicity in the dog.

d. WR 158,122 + WR 7557 (10:1 ratio)

When these two drugs, in combination, were administered intravenously in doses of 25 mg/kg and 2.5 mg/kg respectively, no significant changes were noted in any of the parameters measured.

3. Hemolysis by 8-aminoquinolines.

The hemolytic effect of the 8-aminoquinolines on washed normal human red blood cells was studied in vitro to assess the potential degree of this form of toxicity to humans. Six different drugs were tested: WR 2975 (Primaquine), WR 4234 (Pamaquine), WR 183,538, WR 181,441, WR 181,450 and WR 182,471. The drugs, in concentrations of 0.02, 0.065 and 0.10 mg/ml, were incubated in saline with red cells at 37°C. After centrifugation the optical density of the hemolysate was determined, using appropriate standards and blanks. All of the drugs, with the exception of pamaquine, proved to be more hemolytic than primaquine at 0.1 mg/ml. At 0.065 mg/ml WR 181,450 showed less hemolysis than primaquine. WR 183,538 was extremely hemolytic at all three drug concentrations while the remaining five drugs all seemed to have similar hemolytic effects at 0.02 mg/ml. More sensitive methods are being employed to study lower drug concentrations.

4. Modification of host drug-metabolizing capacity.

a. Background:

Antimalarial agents, like many other chemicals and drugs, are known to be biochemically altered by a variety of processes in the animal body. When this occurs, the efficacious, toxicological and pharmacological effects can be significantly influenced. The various desirable or undesirable properties of a drug may not be those of the administered parent chemical but rather those of its metabolite(s). Such aspects may bear importantly on the clinical value of new antimalarials being readied for trial in man, this value being determined as much by toxicity as by antimalarial potency. Consequently, it is important to determine, as part of the preclinical testing program, if candidate antimalarials undergo

metabolism by the mammalian host so as to affect toxicity and efficacy. The investigative challenge then, is to devise animal model systems that can provide some insight into these and related questions despite the technical obstacles and inevitable associated interpretational complexities.

Our objective is to develop and to exploit animal models for study of the impact that pharmacokinetic factors may have on toxicity and efficacy of these drugs. From these studies we hope to provide a basis for predicting some of the response of humans to antimalarials in a military environment and to help fulfill requirement for submission of IND's for clinical trials of new antimalarials.

In last year's Annual Progress Report we described a new mouse model designed as a presumptive test for in vivo evaluation of the potential roles of host drug-metabolizing enzymes in the toxicity (uninfected mice) and efficacy (malarious mice) of anti-malarial agents. Many of these enzymes are either induced by phenobarbital or inhibited by SKF 525A. An observed change in the toxicity and/or in the parasitemia-suppressing potency of an antimalarial after such pretreatment is taken as presumptive evidence of the participation of host drug-metabolizing enzymes. Results may point to the existence of active metabolites. The usefulness of this experimental approach has been further broadened by expanded testing of candidate antimalarials.

Results from the initial exploration of another potentially useful in vivo test system are also given. It entails the characterization, by phase contrast microscopy, of the morphology of hemozoin pigment granules in endoerythrocytic malarial parasites in response to treatment of the infected mouse with antimalarials and other agents.

b. Methods:

Female CD-1 mice, 7-9 weeks old, were injected intraperitoneally (i.p.) with freshly prepared inocula of red cells infected with the drug-sensitive KBG 173 strain of P. berghei. Approximately 5×10^5 parasitized erythrocytes were injected into each mouse, producing an experimentally suitable prolonged course of infection resulting in death. Antimalarial agents were given once orally by stomach tube as a solution or suspension in 0.9% saline containing 0.4% Tween 80 and 0.2% methyl cellulose (MCTW). Control animals received the vehicle only

Liver microsomal enzymes were induced by 3 daily (Days 0, 1, 2) i.p. injections of phenobarbital sodium (100 mg/kg) dissolved in water. We have found that this phenobarbital pretreatment regimen

reduces hexobarbital-induced sleeping time from 30 minutes to about 5 minutes. The first phenobarbital treatment was given 1 hour before antimalarial parasite inoculation (Day 0) and the antimalarial drugs were given on Day 3, one day after the third phenobarbital injection. The antimalarials were routinely given in suppressive but not curative doses.

To produce inhibition of hepatic drug-metabolizing enzymes, SKF 525A (50 mg/kg) was given once i.p. 45 to 60 minutes before oral antimalarial drug treatment on Day 3. Controls received i.p. saline only. This SKF 525A pretreatment caused prolongation of hexobarbital sleeping time to 4 hours in mice dosed as above. Ordinarily by 24 hours, however, this inhibitory effect is reversed, being supplanted by a state of enzyme stimulation.

Parasitemia was determined on Days 3, 7, 10 and 14 of infection by examination of at least 250 RBC in Giemsa-stained thin blood films and is expressed as percentage of cells parasitized. On these days, smears were taken 1-2 hours before the daily mortality check. The Day 3 smear was taken before antimalarial treatment. Mortality was routinely followed daily for 14 days or longer if necessary.

The main criterion used for judging that the pretreatment regimens affected the parasitemia-suppressing action of the test antimalarial is a statistically significant difference in parasitemia levels between the antimalarial-treated mice which were pretreated with phenobarbital or SKF 525A and antimalarial-treated mice which were pretreated with vehicle only. Comparison of parasitemia levels between the later mice and those receiving no antimalarial, shows the degree of parasitemia suppression effected by the antimalarial at the ordinarily subcurative dose level purposely used.

c. Results and discussion:

i. Effect of phenobarbital or SKF 525A pretreatment on acute oral toxicity of primaquine

Phenobarbital pretreatment afforded mice significant protection against the lethal effects of a large, single dose of primaquine. The magnitude of this protection and the effects of SKF 525A pretreatment on the LD₅₀'s were determined and compared in uninfected mice. Primaquine in MCTW was administered once by stomach tube either 1 day after the third injection of phenobarbital or 1 hour after SKF 525A. Cumulative mortality for 7 days after primaquine was the parameter used to estimate the LD₅₀ by probit analysis of quantal response according to Finney.

The results show that the LD₅₀ of primaquine for mice pretreated with phenobarbital was more than twice that for the vehicle controls and almost twice that for the SKF 525A-pretreated mice. The figures, in mg/kg, for the means and 95% confidence limits were 337 (305-373) for primaquine, 712 (615-826) for phenobarbital pretreatment and 396 (335-469) for SKF 525A pretreatment. The reduction of primaquine lethality by phenobarbital pretreatment suggests that drug-metabolizing enzymes serve to detoxify primaquine and/or its metabolites. The SKF 525A results suggest that this inhibitor does not affect the phenobarbital inducible enzymes responsible for the effects observed with primaquine. This tentative interpretation requires further study, however.

ii. Effect of phenobarbital or SKF 525A pretreatment on the efficacy of candidate antimalarial agents

The results obtained from the screening of nine candidate antimalarials are shown in Table 1 and are summarized in Table 2. Neither phenobarbital nor SKF 525A by themselves altered the course of *P. berghei* infection. All of the agents (Table 2) show a significant alteration in antimalarial activity in response to the phenobarbital and/or SKF 525A pretreatments. This is interpreted as meaning that all undergo metabolism in the mouse. Phenobarbital pretreatment (Table 2) had no effect on the activity of two agents, caused a decrease in six, and an increase in one. SKF 525A pretreatment resulted in increases in activity of eight of the agents and had no effect on the remaining one.

Several distinctive patterns of change in antimalarial activity after phenobarbital and SKF 525A pretreatments were observed. The most common one, exhibited by five of the nine agents, was a decrease after phenobarbital and an increase after SKF 525A. This particular pattern strongly suggests that: metabolism serves to terminate the antimalarial activity of these agents; that parent chemical, not a metabolite, is responsible for antimalarial action; interactions between these agents and other medications that may affect drug metabolism are a distinct clinical possibility; enzyme-inducers such as phenobarbital might be useful in helping rid the body of these agents when the therapeutic effects are no longer required; and in view of the results with SKF 525A, structural modification of these agents at possible sites susceptible to metabolic attack may be helpful in increasing their antimalarial potency.

In the case of WR 33,063, both phenobarbital and SKF 525A increased its antimalarial activity. The increase in efficacy produced by SKF 525A was significantly greater than that elicited by phenobarbital (Table 1) and resulted in four cures, no cures being seen in any of the other parallel groups. The phenobarbital-induced increase suggests that a more potent metabolite is produced

by the host. The still greater increase in antimalarial activity produced by SKF 525A might be interpreted to signify that the metabolism of the active metabolite, but not the metabolism of the parent compound, may have been inhibited. This interpretation allows that the parent compound may itself possess some antimalarial activity and may contribute to the result.

It is interesting that the sulfide form of the thio-quinazoline WR 154,928 and its corresponding sulfonyl analogue WR 158,122 exhibited similar antimalarial activities (Table 3) and identical responses after the phenobarbital and SKF 525A pretreatment (Table 1), viz., no change in efficacy after the former and an increase after the latter. If phenobarbital affects the enzymes involved in the metabolism of these agents, the metabolites are neither more nor less potent than the parent chemical. However, the increased activity produced by SKF 525A pretreatment indicates that the two pretreatments do not affect all of the same enzyme systems that act on the drug.

WR 122,455 was the only drug that did not exhibit an increase in antimalarial activity after SKF 525A pretreatment, but did show a decrease in activity after phenobarbital. These findings suggest that the parent compound is the active antimalarial and that its metabolism, apparently stimulated by phenobarbital pretreatment, is not inhibited by SKF 525A.

The effect of micronization on the suppressive efficacy of WR 159,412 was investigated. No statistically significant biological difference was seen between micronized and unmiconized drug at 5 mg/kg.

In summary, our experience to date with this test system affirms its feasibility and utility. Results obtained, taken together with other types of information, may prove especially valuable in helping to single out which of the prospective new antimalarials may merit more detailed, in depth metabolic evaluations.

d. Effect of certain antimalarial agents on hemozoin pigment morphology:

Warhurst and coworkers (1971a, 1971b) have shown that chloroquine induces a coalescence of hemozoin pigment in malaria parasites in vivo, an effect which can be inhibited by i.p. pretreatment with certain antimalarials. While this technique is now apparently being pursued exclusively in vitro by that group, we have elaborated on various in vivo aspects by modifying the procedure. For example, we are using oral administration to yield information appropriate for inclusion in an IND. This would

include evidence of absorption from the GI tract and entry into the parasite. The effect of various vehicles, formulations and/or drug combinations on absorption can also be studied in this system.

We found that a dose of 40 mg/kg of chloroquine i.p. caused no deaths and induced clumping to an extent satisfactory for its establishment as the standard dose in our mice (Charles River, CD-1, females).

Eighty minutes after chloroquine, phase microscopic examination of unfixed, unstained thin films of blood from infected mice revealed clumps of fully aggregated pigment (Type III morphology) in a population of our P. berghei (the drug-sensitive KBG 173 strain). At shorter time intervals, e.g., 30-40 minutes after chloroquine, some parasites had fully aggregated pigment clumps, while other demonstrated only a partial aggregation of pigment (Type II morphology). We refer to this combination in a microscope field of both total and partial aggregation of pigment as partial clumping. Prior to chloroquine, smaller pigment granules were dispersed more evenly throughout the parasite (Type I morphology). These observations indicate a transformation after chloroquine from fine pigment through a transitional granular type, to a final clumped appearance, which persisted at least 6 hours.

Mice inoculated with 1×10^7 parasitized RBC showed an experimentally suitable parasitemia of approximately 15% on Day 3. The test drug was administered before chloroquine and the effect on C-IPC (chloroquine-induced pigment clumping) was determined by examination of thin blood films (50 parasitized RBC) taken at intervals after chloroquine. The percentage distribution of the three pigment types is compared to determine the degree of test drug-induced alterations, if any.

We confirmed that i.p. administration of WR 33,063 or WR 30,090 caused inhibition of C-IPC (Warhurst et al., 1972). To study shorter intervals between drug pretreatment and chloroquine administration in our exploration of the oral effect of several agents, we administered test drugs as soon as 30 minutes before chloroquine and examined pigment morphology as early as 30 minutes after chloroquine. As mentioned above, a typical microscope field reveals partial clumping of pigment 30 or 40 minutes after chloroquine. Inhibition (either partial or total) of this partial clumping may be detected by comparison with groups receiving chloroquine alone. This differentiation is useful not only in demonstrating gradations of effect but also may be used to quantitate the time course of drug absorption.

Table 4 illustrates our results with WR 30,090 and WR 33,063, both of which had a direct effect on the parasite within a total time of 60 minutes. WR 30,090 at 10 mg/kg had a greater effect than 640 mg/kg of WR 33,063 after a total time of 60 minutes. Neither of these agents themselves caused pigment clumping in the doses studied. Thus, it appears that by 1 hour enough of each drug, when given by gavage, had been absorbed to affect the parasite directly.

An attempt was made to compare two formulations of WR 30,090, one with Cab-O-sil vs. the corresponding preparation containing no Cab-O-sil. Table 4 illustrates that all test doses were too well absorbed to demonstrate a difference in bioavailability between the two formulations. Doses lower than 10 mg/kg will have to be used to yield data allowing a distinction.

SKF 525A alone does not alter the course of P. berghei infection. However, since we very often find increased efficacy of antimalarials (8 of 9 compounds tested so far, Table 2) after pretreatment of infected mice with SKF 525A, we have considered the possibility that SKF 525A may have some subtle direct effect on the parasite in addition to its routinely-used inhibitory effect on host biotransformation enzymes. We therefore looked for an effect of SKF 525A both on pigment morphology and on C-IPC. We have found that 50 mg/kg i.p. of SKF 525A given alone does not induce pigment coalescence within 450 minutes. In addition, the compound does not inhibit C-IPC when given 90 minutes before chloroquine, even when pigment morphology is followed up to 360 minutes after chloroquine. Therefore, we could not demonstrate any effect of SKF 525A on a population of parasites by our method.

Table 1
Effect of Phenobarbital or SKF 525A Pretreatment on the Antimalarial Activity
of Various Antimalarials in Mice Infected with P. berghei^a

Oral Therapy on Day 3			Responses to Therapy ^b			
Agent	Dose (mg/kg)	Pre- treatment ^c (i.p.)	Day 3	Day 7	Day 10	Day 14
MCTW ^d						
	-	P, S, or V ^d	2.2(436) ^d	69.4(258) ^d	- (36) ^d	- (20) ^d
<hr/>						
WR 148,946 AD	5	V	2.0(36)	25.0(34)	40.6(26)	52.6(18)
		P	2.0(36)	45.4(18) ^e	43.2(3)	- (0)
		S	2.0(12)	13.8(12) ^e	45.0(12)	59.6(7)
WR 30,090 AJ	5	V	2.4(24)	0.0(24)	12.2(24)	- (0)
		P	2.4(22)	1.0(22) ^e	16.4(13)	37.0(4)
		S	2.0(24)	0.0(24)	0.0(24) ^e	26.8(12)
WR 142,490 AE	20	V	2.2(12)	0.8(12)	0.8(12)	2.6(12)
		P	2.0(12)	34.6(12) ^e	44.8(6) ^e	62.8(4) ^e
	5	V	2.0(20)	27.0(20)	53.2(13) ^e	65.0(8)
		S	2.4(20)	7.2(20) ^e	38.2(16) ^e	62.4(13)
WR 159,412 AD	5	V	2.4(23)	5.6(23)	38.0(15)	54.4(5)
		P	2.8(22)	22.4(21) ^e	35.4(19)	54.8(15)
		S	2.0(23)	1.2(23) ^e	11.2(23) ^e	41.8(4)
WR 158,122 AC	20	V	2.0(36)	2.0(35)	15.2(35)	43.2(23)
		P	2.4(36)	4.2(36)	20.4(35) ^e	43.6(32)
	10	V	2.4(12)	7.0(12)	20.6(12)	47.0(8)
		P	2.4(12)	8.8(12)	12.0(12) ^e	51.6(9)
		S	2.0(12)	2.8(11) ^e	3.2(11) ^e	19.2(5) ^e

Table 1 (continued)

Oral Therapy on Day 3		Responses to Therapy ^b				
Agent	Dose (mg/kg)	Pre-treatment ^c (i.p.)	Day 3	Day 7	Day 10	Day 14
WR 154,928 AE	10	V	2.4(12)	3.8(12)	17.2(11)	42.4(7)
		P	2.4(12)	3.0(12)	9.6(12)	32.2(10)
		S	2.0(12)	1.6(11) ^e	2.0(11) ^e	24.8(7)
WR 33,063 AE	160	V	2.0(36)	0.8(35)	24.8(27)	59.6(11)
		P	2.0(36)	0.0(36) ^e	1.2(33) ^e	23.0(10) ^e
		S	1.6(22)	0.0(22) ^e	0.0(22) ^e	5.2(22) ^e
WR 171,669 AC	2.5	V	2.2(24)	0.8(24)	24.4(17)	58.8(3)
		P	2.4(24)	10.2(22) ^e	40.4(21) ^e	58.4(19)
		S	2.0(24)	0.0(24)	5.4(24) ^e	26.4(3)
WR 122,455 AF	5	V	2.8(24)	6.8(23)	33.2(21)	48.4(11)
		P	2.8(23)	17.2(23) ^e	42.4(21)	45.6(10)
		S	2.0(24)	4.0(24)	37.8(22)	43.2(14)

^a All mice received 5×10^5 parasitized RBC i.p. on Day 0.

^b Median parasitemia as % parasitized RBC, based on number of surviving mice shown in parentheses.

^c Mice received phenobarbital sodium, 100 mg/kg (P) for 3 days prior to therapy (Days 0,1,2) or one injection of SKF 525A, 50 mg/kg (S) on Day 3, 1 hour before therapy, or the respective vehicle (V), water or 0.9% saline, both at 1% body weight. Both vehicles gave identical results and, therefore, are not considered separately.

^d Controls that received the vehicle MCTW, 1% of body weight, irrespective of type of pretreatment (P,S, or V) showed identical results which are here pooled. Each value represents the overall mean of the medians of % parasitemia of 42 P, S, and V groups from 18 experiments (486 mice). Because of high mortality, median values (based on 3 or more survivors) were rare on Days 10 and 14 among the 42 groups pooled. Therefore, no values are given on these days.

^e Difference from corresponding vehicle control group (V) is statistically significant ($p < 0.05$ by Wilcoxon's Rank Test).

Table 2

Effect of Phenobarbital or SKF 525A Pretreatment on the Parasitemia-Suppressing Potency of Various Antimalarials in Mice^a

Oral Therapy on Day 3 of Infection		Change in Parasitemia Suppression after Pretreatment with:	
Agent (general structure)	Dose (mg/kg)	Phenobarbital ^b	SKF 525A ^b
WR 148,946 (pyridine methanol)	5	Decrease	Increase
WR 30,090 (quinoline methanol)	5	Decrease	Increase
WR 142,490 (quinoline methanol)	20 5	Decrease (c)	(c) Increase
WR 159,412 (quinazoline)	5	Decrease	Increase
WR 158,122 (quinazoline)	20 10	None None	(c) Increase
WR 154,928 (quinazoline)	10	None	Increase
WR 33,063 (phenanthrene methanol)	160	Increase	Increase ^d
WR 171,669 (phenanthrene methanol)	2.5	Decrease	Increase
WR 122,455 (phenanthrene methanol)	5	Decrease	None

^aAll mice given 5×10^5 infected RBC i.p. on Day 0.

^bRelative to that observed in infected mice receiving antimalarial but no phenobarbital or SKF 525A.

^cExperiment not done.

^dThe increase in antimalarial activity was unequivocally greater after SKF 525A than after phenobarbital.

Table 3

Lack of Effect of the State of Sulfur Oxidation on the Antimalarial Activity of Two Thioquinazoline Sulfides and Their Corresponding Sulfones in Mice Infected with P. berghei^a

Oral Therapy on Day 3		Structure Compared	Responses to Therapy ^b			
Agent ^c	Dose (mg/kg)		Day 3	Day 7	Day 10	Day 14
WR 158,122 AC	20	Sulfonyl	2.0(12)	0.0(12)	14.8(12)	34.2(10)
	10		2.4(12)	7.0(12)	20.6(12)	46.4(8)
WR 154,928 AE	20	Sulfide	2.0(11)	0.0(11)	9.6(11)	18.4(7)
	10		2.4(12)	3.8(12)	17.2(11)	42.4(7)
WR 162,878 AB	10	Sulfonyl	1.8(10)	1.2(10)	5.4(10)	29.2(5)
WR 159,412 AD	10	Sulfide	2.4(10)	1.4(10)	9.4(10)	38.8(5)

^aAll mice received 5×10^5 parasitized RBC i.p. on Day 0.

^bMedian parasitemia as % parasitized RBC, based on number of surviving mice shown in parentheses. None of the apparent differences between comparison groups is statistically significant ($p > 0.05$ by Wilcoxon's Rank Test).

^cAll agents suspended in MCTW.

Table 4

Effects of Antimalarials on Chloroquine-Induced Pigment Clumping

Drug	Oral Dose (mg/kg)	Number of mice	t ₁ ^a (min)	t ₂ ^a (min)	Total Time ^a (min)	Results ^b
WR 33,063 AN	640	5	360	80	440	A
"	"	3	"	40	400	B
"	"	3	180	"	220	B
"	"	7	30	30	60	D
"	"	"	"	80	110	C
"	160	"	"	30	60	D
"	"	"	"	80	110	C
WR 30,090 AJ	80	7	30	30	60	B
"	"	"	"	80	110	A
WR 30,090 AH (No Cab-o-sil)	10	"	30	30	60	B
"	20	"	"	"	"	B
WR 30,090 AH (20% Cab-o-sil)	10	5	30	30	60	B
"	20	"	"	"	"	B

^a Test agents were administered at time t₁ before chloroquine and pigment morphology was examined at time t₂ after chloroquine. Total time, the sum of t₁ and t₂, is the period elapsed after administration of test agent.

^b Interpretations are based on comparisons with appropriate control groups, e.g. chloroquine alone, vehicle controls, etc. Symbols in the results column refer to the following:

A - Total inhibition of total clumping, B - Total inhibition of partial clumping,
C - Partial inhibition of total clumping, D - Partial inhibition of partial clumping.

^c Doses are expressed in terms of W₀ 30,090 AH.

Summary and Conclusions

The use of human malarial parasites-both P. falciparum and P. vivax-in standardized animal systems continues to permit selection and chemical development of new structure leads which do not show cross resistance against human strains of interest. Clinical and field evaluation of these candidate antimalarials is progressing at a rapid pace. Preclinical studies during this year have resulted in the submission and approval of 4 new Investigational New Drug Applications and 15 Supplements. Promising new chemical leads have been uncovered that await development as therapeutic and prophylactic antimalarials. The status of the Antimalaria Drug Development Program supports the recommendation that research and development effort be continued at the present level. In-depth evaluation of new chemicals and efforts to improve the bioavailability of our present clinical drugs are necessities. Program changes have been made to emphasize these needs.

Project 3A663713D829 MALARIA PROPHYLAXIS

Task 00, Malaria Investigations

Work Unit 134 Malaria Investigations

Literature Cited.

References:

1. Warhurst, D. C. and Robinson, B. L.: Cytotoxic agents and haemozoin pigment in malaria parasites (Plasmodium berghei). Life Sciences 10: 755-760, 1971a.
2. Warhurst, D. C., and Robinson, B.L., Howells, R. E. and Peters, W.: The effect of cytotoxic agents on autophagic vacuole formation in chloroquine-treated malaria parasites (Plasmodium berghei). Life Sciences 10. 761-771, 1967b.
3. Warhurst, D. C. , Homewood, C. A., Peters, W. and Baggaley, V. C.: Pigment changes in Plasmodium berghei as indicators of activity and mode of action of antimalarial drugs. Proc. Helminth. Soc. Wash. 39: 271-278, 1972.

Publication:

1. Rozman, R. S.: Chemotherapy of malaria. Ann. Rev. Pharmacol., 13: 127-152, 1973.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION ^a	2. DATE OF SUMMARY ^a	REPORT CONTROL SYMBOL	
				DA OA 6535	72 08 01	DD-DR&E(AR)636	
3. DATE PREV SUMMARY	4. KIND OF SUMMARY	5. SUMMARY SCTY ^a	6. WORK SECURITY ^a	7. REGRADING ^a	8A. DISSEM INSTR ^a	8B. SPECIFIC DATA CONTRACTOR ACCESS ^a	9. LEVEL OF SUM
72 07 01	H.Termination	U	U	NA	NL	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	A. WORK UNIT
10. NO./CODES ^a		PROGRAM ELEMENT		PROJECT NUMBER		TASK AREA NUMBER	
A. PRIMARY		63713A		3A663713D829		00	
B. CONTRIBUTING						136	
C. CONTRIBUTING		CDOG 114(F)					
11. TITLE (Precede with Security Classification Code) ^a							
(U) Metabolic and Enzymatic Studies of Normal and Malarial Infected Red Cells							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS ^a							
002600 BIOLOGY							
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
66 12		72 08		DA		C. In-House	
17. CONTRACT/GRANT NA				18. RESOURCES ESTIMATE		19. PROFESSIONAL MAN YRS	
A. DATES/EFFECTIVE: EXPIRATION				PRECEDING			
B. NUMBER ^a				FISCAL YEAR		2	
C. TYPE: & AMOUNT:				CURRENT		60	
D. KIND OF AWARD: F. CUM. AMT.				73		2	
19. RESPONSIBLE DOD ORGANIZATION				20. PERFORMING ORGANIZATION			
NAME ^a Walter Reed Army Institute of Research				NAME ^a Walter Reed Army Institute of Research			
ADDRESS ^a Washington, D.C. 20012				ADDRESS ^a Division of Medicine			
				Washington, D.C. 20012			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)			
NAME: Buescher, COL E. L.				NAME ^a McCormick, G. J., Ph.D.			
TELEPHONE: 202-576-3551				TELEPHONE: 202-576-2497			
21. GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER			
Foreign Intelligence Not Considered				ASSOCIATE INVESTIGATORS			
				NAME:			
				NAME:			
				DA			
22. KEYS (Precede EACH with Security Classification Code)							
(U) Malaria; (U) Antimalarials; (U) Parasite; (U) Red Blood Cell							
23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
23. (U) Document metabolic alterations of human and animal red blood cells when infected with malaria parasites and to assess the effect of antimalarial drugs on these alterations in order to develop new drugs effective against resistant falciparum malaria.							
24. (U) Measure the effect of antimalarial drugs on morphologic growth, 14-C adenosine 14-C methionine or 14-C orotic acid incorporation during in vitro schizogony and observe utilization of metabolic precursors of nucleic acids; to measure folic acid reductase in parasite suspensions.							
25. (U) 72 07 - 72 08 This work Unit terminated as of 1 Aug 72 because of consolidation into another work unit. Studies to be continued under Project No. 3A663713D829, Work Unit No. 132.							

DD FORM 1 MAR 66 1498

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PII Redacted

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY					1. AGENCY ACCESSION ^a	2. DATE OF SUMMARY ^a	REPORT CONTROL SYMBOL CD-DR&E(AR)836	
3. DATE PREV SUMMARY ^a	4. KIND OF SUMMARY ^a	5. SUMMARY SCTY ^a	6. WORK SECURITY ^a	7. REGRADING ^a	8. DISSEM INSTN ^a	9. SPECIFIC DATA: CONTRACTOR ACCESS <input checked="" type="checkbox"/> YES <input type="checkbox"/> NO		10. LEVEL OF SUM A. WORK UNIT
72 07 01	H. TERM.	U	U	NA	GP			
10. NO./CODES ^a		PROGRAM ELEMENT		PROJECT NUMBER		TASK AREA NUMBER		WORK UNIT NUMBER
a. PRIMARY		63713A		3A663713D829		00		171
b. CONTRIBUTING								
c. CONTRIBUTING		CDOG 114 (F)						
11. TITLE (Precede with Security Classification Code) ^a (U) General Pharmacology of Antimalarial Drugs (09)								
12. SCIENTIFIC AND TECHNOLOGICAL AREAS ^a 012600 Pharmacology								
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD		
70 07		72 08		DA		In-House		
17. CONTRACT/GRANT				18. RESOURCES ESTIMATE		19. PROFESSIONAL MAN YRS		20. FUNDS (In thousands)
a. DATES/EFFECTIVE: NA				EXPIRATION:		PRECEDING		
b. NUMBER: ^a				c. TYPE:		FISCAL YEAR		
d. KIND OF AWARD:				e. CUM. AMT.		72		8 350
						73		8 350
21. RESPONSIBLE DOD ORGANIZATION				22. PERFORMING ORGANIZATION				
NAME: ^a Walter Reed Army Institute of Research Washington, D. C. 20012				NAME: ^a Walter Reed Army Institute of Research Division of Medicinal Chemistry Washington, D. C. 20012				
ADDRESS: ^a				ADDRESS: ^a				
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)				
NAME: BUESCHER, COL, E. L.				NAME: ^a Melvin H. Heiffer, Ph.D.				
TELEPHONE: 202/576-3551				TELEPHONE 202/576-3387				
				SOCIAL SECURITY ACCOUNT NUMBER [REDACTED]				
23. GENERAL USE				ASSOCIATE INVESTIGATORS				
Foreign Intelligence Not Considered				NAME: Einheber, A., Ph.D.				
				NAME: Rozman, E., Ph.D.				
24. KEYWORDS (Precede EACH with Security Classification Code) (U) Pharmacodynamics; (U) Pharmacokinetics; (U) Toxicity (U) Drug Metabolism; (U) Antimalarial Drugs; (U) Preclinical Pharmacology								
25. TECHNICAL OBJECTIVE, 26. APPROACH, 27. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)								
23. (U) The technical objective is to develop and to exploit animal models for the study of the pharmacodynamics and toxic effects of drugs intended for use as antimalarials in man. The intended purpose of these studies is to provide a basis for predicting the response of soldiers to antimalarials in a military environment and to fulfill requirements for submission of IND for clinical trials of new antimalarials.								
24. (U) The approach will be to study the effects of antimalarial drugs in healthy animals and to study the handling of antimalarial drugs in healthy animals in order to predict the human tolerance to new drugs (Phase I). The effects of antimalarial drugs in diseased or injured animals will be studied in order to determine the effects of the drugs on disease and injury. The handling of antimalarial drugs by diseased and injured animals will be studied in order to determine the effect of disease or injury upon pharmacokinetics in order to predict the tolerance of new antimalarial drugs in human efficacy studies (Phase II).								
25. (U) Studies formerly reported under this work unit are reported under 3A663713D829, Work Unit Number 134.								

^aAvailable to contractors upon originator's approval

DD FORM 1498

PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A 1 NOV 65 AND 1498-1 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE

1187

PII Redacted

PROJECT 3A062110A830
BIOSENSOR SYSTEMS

Task 00
Biosensor Systems

1188

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY					1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL DD-DR&E(AK)836	
3. DATE PREV SUMMARY	4. KIND OF SUMMARY	5. SUMMARY SCT	6. WORK SECURITY	7. REGRADING	8A. DISSEM INSTR	8B. SPECIFIC DATA CONTRACTOR ACCESS		9. LEVEL OF SUM A. WORK UNIT
72 07 01	D. Change	U	U	NA	NL	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO		
10. NO / CODES	PROGRAM ELEMENT	PROJECT NUMBER		TASK AREA NUMBER		WORK UNIT NUMBER		
A. PRIMARY	62110A	3A062110AB30		00		055		
B. CONTRIBUTING								
C. XXXXXXXX	CD0G 114(f)							
11. TITLE (Precede with Security Classification Code)								
(U) Development and Evaluation of Improved Biological Sensor Systems (21)								
12. SCIENTIFIC AND TECHNOLOGICAL AREAS								
001700 Animal Husbandry 01180 Operations								
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD		
67 09		Cont		DA		C. In-House		
17. CONTRACT/GRANT				18. RESOURCES ESTIMATE		A. PROFESSIONAL MAN YRS		B. FUNDS (In thousands)
A. DATES/EFFECTIVE: NA				PRECEDING		3.25		260
B. NUMBER				FISCAL YEAR		CURRENT		
C. TYPE				73		74		260
D. AMOUNT:				74		3.5		
E. KIND OF AWARD:				F. CUM. AMT.				
19. RESPONSIBLE DOD ORGANIZATION				20. PERFORMING ORGANIZATION				
NAME: Walter Reed Army Institute of Research				NAME: Walter Reed Army Institute of Research				
ADDRESS: Washington, DC 20012				Division of Bio Sensor Research				
				ADDRESS: Aberdeen Proving Ground, MD 21010				
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)				
NAME: Buescher, COL E.L.				NAME: Castleberry, COL. M.W.				
TELEPHONE: 202-576-3551				TELEPHONE: 301-671-3312				
				SOCIAL SECURITY ACCOUNT NUMBER: [REDACTED]				
21. GENERAL USE				ASSOCIATE INVESTIGATORS				
Foreign intelligence not considered				NAME: Lees, CPT G.E.				
				NAME: Hamlin, CPT M.H.				
				DA				
22. KEYWORDS (Precede EACH with Security Classification Code)								
(U) Detector System; (U) Dogs; (U) Genetics; (U) Selective Breeding								
23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)								
23. (U) To develop a more intelligent and sensually acute dog which is physically and temperamentally better suited for military purposes than is now generally available.								
24. (U) This study is being made in response to the approved (11 Dec 68) US Army QMDO, Detector System Military Dog (USACDC Action Control Number 12527). Critically evaluated AKC registered dogs were purchased as foundation stock. The progeny of these are closely evaluated by recognized tests designed to reveal the superior individual.								
25. (U) 72 07 - 73 06 Forty-two litters produced 246 weaned puppies. Present kennel population is 232 German Shepherd Dogs. During the year 257 dogs were transferred to other Federal activities including the DOD Dog Center at Lackland AFB, WRAIR, Ft. Monmouth, and the State Department. An additional 17 dogs were retained as breeders. Puppies born during the past year are generally more willing and aggressive than heretofore. Fourteen of 34 FY73 litters which have completed puppy evaluation are considered superior in this respect. Only 4 of the 45 litters produced during FY72 were comparable to those herein reported. This improvement in temperament is attributed primarily to the selective breeding program. This Division is cooperating with the Letterman Army Institute of Research in its recently initiated study of canine eosinophilic panosteitis. Consultant visits were made to this Division by Dr. W.H. Riser (hip dysplasia); Dr. M.W. Fox (canine behavior); Dr. D.F. Patterson (genetics) and Mr. E.H. Hart (breeding and blood lines). For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 72 - 30 Jun 73.								

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DD FORM 1498
1 MAR 68

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1188 a

Project 3A062110A830 BIOSENSOR SYSTEMS

Task 00 Biosensor Systems

Work Unit 055 Development and evaluation of improved biological sensor systems

INVESTIGATORS.

Principal - COL Merida W. Castleberry, VC
Associates - CPT George E. Lees, VC
CPT Melvin H. Hamlin II, VC

OBJECTIVE. To develop a more intelligent and sensually acute dog which is physically and temperamentally better suited for military purposes than is now generally available.

BACKGROUND. This study is being made in response to the approved US Army QMDO, "Detector System, Military Dog", (USACDC Action Control Number 12527). Seven breeds of dogs, including crosses, were studied by the University of Maryland for behavioral evaluation and selection for army breeding and training (Army Contract No. DADA 17-68-C-8015). As recommended in the final report of that study, and because of the years of military experience gained with the German Shepherd Dog, this breed was selected for primary breeding emphasis.

APPROACH. Critically evaluated AKC registered breeding stock purchased especially for this purpose was selectively bred to produce superior progeny. These are in turn closely evaluated by recognized tests designed to reveal the superior individual. Line breeding combined with progeny testing of each generation is being used to accomplish the objective.

PROGRESS.

A. Breeding Program

1. Forty-two litters produced 246 weaned puppies.
2. Present kennel population is 232 dogs.
3. Two hundred and fifty-seven dogs were transferred to other Federal activities. These were:

Walter Reed Army Institute of Research	183
Patterson Army Hospital, Ft. Monmouth, NJ	12
DOD Dog Procurement Center, Lackland AFB, TX	47
Biomedical Laboratory, APG-EA, MD	5
State Department	4
USA Mobility Equipment Rsch & Dev Center	4
Ft. Gordon, GA	1
US Customs	1

4. Seventeen dogs were selected and retained as additions to the breeding colony.

B. Special Projects

1. Collection of data to determine the possible relationship of canine heart rates and adrenal-pituitary function to natural aggressiveness and self-confidence was completed. The study is composed of 93 puppies from 19 different litters. Heart rates were determined and blood samples collected from these puppies during their twelfth week of life. Early difficulties in the telemetering of heart rates were overcome and blood collecting techniques standardized. Interpretation of the individual heart rates and plasma cortisol values to the aggressiveness and confidence subsequently exhibited by each test puppy as it matures will be accomplished at the time of its shipment or designation as a breeder.

2. One of the primary rejection factors in the procurement and training of military working dogs is that of underaggressiveness. In conjunction with the USA Land Warfare Laboratory an exploratory study was recently initiated to determine the efficacy of food reinforcement in the correction of this trait. This is accomplished by positive reinforcement of an on-going activity which is suddenly discontinued. An aggressive response by the dog to the sudden cessation of the food gathering activity (key pressing) is rewarded by reactivation of the activity. Otherwise he receives a limited time-out period during which he receives no further food. Instrumentation has been completed and pre-experimental baseline data is now being collected. Further work is contingent on the nature of results from this exploratory study.

3. Eosinophilic panosteitis is one of several synonyms that identify an idiopathic clinical syndrome that is characterized by intermittent lameness seen in larger breeds of dogs. It is most frequently reported in the German Shepherd Dog. The condition has been radiographically identified several times in individual dogs of this kennel. This organization is cooperating with the Veterinary Division, Letterman Army Institute of Research in their recently initiated study to define the biological parameters, radiographic findings, and etiology of eosinophilic panosteitis of dogs. Upon diagnosis, effected dogs with medical records including radiographs will be sent to the LAIR.

C. Veterinary Medicine.

This Division continued its vigorous veterinary preventative medicine program. Its canine vaccination program is only one aspect of the effort. Starting with the puppy's fourteenth day of life, measles vaccination is administered to provide an early cross immunity protection to distemper. Distemper and hepatitis vaccine is administered at eight weeks of age. A combination distemper, hepatitis and leprospirosis vaccine is administered at eleven and fourteen weeks of age, with boosters annually thereafter. Rabies immunization begins with phenolized bacterin

at twelve weeks of age, modified live virus vaccine at twenty-four weeks of age, with boosters annually thereafter. Attesting to the efficacy of this program is the fact that this kennel has experienced none of these diseases during the past four years. Some problem was experienced, however, with the hepatitis fraction of the combination distemper and hepatitis vaccine used during the second quarter of this report. In a group of approximately 80 puppies vaccinated during that period, approximately one-fourth failed to attain normal growth rate. In addition, an unusual number were affected with varying degrees of iridocyclitis. In two of these the iridocyclitis became so severe that oblation of the effected eye was required. This particular vaccine is no longer used and the adverse reactions have ceased to be a problem.

The reduction of the incidence of hip dysplasia continues to be an integral part of the objectives of this organization. Dogs are routinely radiographed at five, eight and eleven months of age for this condition. The breeding stock, almost without exception, is OFA certified. During the past fiscal year 288 dogs were radiographed, of these 63 were determined to have hip dysplasia.

The surgical capabilities of Bio Sensor Research Division were improved by the addition of a new gas anesthesia machine. In addition to other routine surgical procedures, nine spays were performed. Two young dogs were diagnosed as having persistent right fourth aortic arches. Thoracotomies were performed on both dogs. A patent ductus arteriosus present in one and a ligamentum arteriosum present in the other were successfully isolated, ligated, and transected.

A rigorous program of detection, treatment and elimination of parasites continued to be maintained. Only two external parasites were of any consequence. One of these, demodectic mange, is occasionally encountered and responds well to treatment. The other is the American Dog Tick which is controlled by periodically dipping the dogs in a .03% Lindane solution. Intestinal parasites are detected by programmed fecal examinations. The primary parasites are ascarides and coccidia. Occasionally the presence of giardia or strongyloides is noted. The canine heart worm, Dirofilaria immitis, was detected in one dog by the Knott test.

D. Consultant Visits.

Consultant visits were made to this Division by Dr. W.H. Riser (hip dysplasia); Dr. M.W. Fox (canine behavior); Dr. D.F. Patterson (genetics) and Mr. E.H. Hart (breeding and blood lines).

E. Equipment.

A veterinary anesthesia machine was the only new equipment purchased this year.

DISCUSSION: An unusual number of aggressive self-confident litters were whelped during the past year. Fourteen of the thirty-four FY73 litters which have completed puppy evaluation are considered superior in this respect. In contrast, only four of the forty-five litters whelped in FY72 were comparable. This improvement is attributed primarily to this organization's selective breeding program. From birth, each puppy is subjected to continued critical evaluation. If eventually retained as a breeder, evaluation is continued by the testing of its progeny. Now well into the production of third generation litters, this progress should accelerate with the maturation and mating of the best individuals of these latest litters.

The accurate selection of the best individuals for breeding purposes is not always simple. Following shipment, individual training reports pertaining to each dog are received from the using organization engaged in the training of military working dogs. These reports assist in the selection of future matings. In addition, they identify those males demonstrating outstanding working dog characteristics at the training centers. Yank, son of an originally purchased male and of a mother born in these kennels, is such a dog. Classified as outstanding at the patrol dog training center and following eighteen months of field use, he has been temporarily returned to these kennels for use as a sire. Several of his litters are now being evaluated.

CONCLUSION. Selective breeding has proven successful in the production of more eggs, milk, and wool. It is proving equally successful in the development of a more stable, smarter, and physically better dog than is now generally available for military use. It is reasonable to expect the development of a line of truly superior military dogs with the next several years.

RECOMMENDATIONS. None.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION ^a	2. DATE OF SUMMARY ^a	REPORT CONTROL SYMBOL
				DA OB 6444	73 07 01	DD-DR&E(AR)636
3. DATE PREV SUMRY	4. KIND OF SUMMARY	5. SUMMARY SCTY ^a	6. WORK SECURITY ^a	7. REGRADING ^a	8A. DESIG ^a INSTR ^a	8B. SPECIFIC DATA - CONTRACTOR ACCESS ^a
72 07 01	D. Change	U	U	NA	NL	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO
9. NO./CODES ^a		10. PROGRAM ELEMENT	11. PROJECT NUMBER	12. TASK AREA NUMBER	13. WORK UNIT NUMBER	
a. PRIMARY		62110A	3A062110A830	00	056	
b. CONTRIBUTING		61101A	3A061101A91C	00		
c. CONTRIBUTING						
11. TITLE (Precede with Security Classification Code) ^a						
(U) Diseases of Military Animals						
12. SCIENTIFIC AND TECHNOLOGICAL AREAS ^a						
010100 Microbiology 005900 Environmental Bio						
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD
68 07		CONT		DA		C. In-House
17. CONTRACT/GRANT				18. RESOURCES ESTIMATE		19. PROFESSIONAL MAN YRS
a. DATES/EFFECTIVE: NA				PRECEDING		b. FUNDS (in thousands)
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d. KIND OF AWARD:				74		180
19. RESPONSIBLE DOD ORGANIZATION				20. PERFORMING ORGANIZATION		
NAME: ^a Walter Reed Army Institute of Research				NAME: ^a Walter Reed Army Institute of Research		
ADDRESS: ^a				Division of Veterinary Medicine		
				Washington, D.C. 20012		
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)		
NAME: Buescher, COL, E. L.				NAME: ^a Huxsoll, LTC, D. L.		
TELEPHONE: 202-576-3551				TELEPHONE: 202-576-5194		
21. GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER: [REDACTED]		
Foreign intelligence not considered.				ASSOCIATE INVESTIGATORS Binn, Ph.D., L. N.		
				NAME:		
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22. KEYWORDS (Precede EACH with Security Classification Code) (U) Military Dogs; (U) Tropical Canine Pancytopenia; (U) Ehrlichia canis; (U) Babesia; (U) Coronavirus; (U) SV5; (U) Canine Respiratory Dis.						
23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)						
23. (U) To define, study, diagnose and control known and potential infectious diseases of military dogs. The major effort is directed toward the cause, pathogenesis, treatment and control of tropical canine pancytopenia (TCP) which jeopardized the operational efficacy of military dogs in SE Asia. Studies are also being conducted on the epidemiology, treatment and control of other viral and parasitic infections which are medical problems in military dogs.						
24. (U) Conventional methods are employed for epidemiological, pathological and microbiological examinations, and new procedures are developed as needed.						
25. (U) 72 07 - 73 06 Clinical response to tetracycline was evaluated in dogs chronically infected with Ehrlichia canis, the causative agent of TCP. In the treated dogs improvement in hematologic values and clinical signs was very gradual. Blood from dogs treated with tetracycline was not infectious for laboratory Beagles. German Shepherd and Beagle dogs experimentally infected with an equine ehrlichial agent developed a mild, transient disease and characteristic inclusions were identified in the cytoplasm of granulocytic cells. The monocyte and bone marrow cell culture techniques were evaluated for the primary isolation of rickettsial agents causing human disease. Initial studies were carried out in guinea pigs experimentally infected with Rickettsia rickettsii. Rickettsiae were readily recognized as early as 3 days in Gimenez and FA stained cultures. Newly procured military dogs at Lackland AFB, Texas continued to be highly susceptible to parainfluenza SV5 infection. Studies were carried out on the pathogenicity of a canine coronavirus isolate from military dogs with diarrheal disease. The virus produced signs of enteric infection in newborn pups. Reovirus type II was recovered for the first time from 2 puppies with respiratory disease. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 72-30 Jun 73.						

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Project 3A062110A830 BIOSENSOR SYSTEMS

Task 00 Biosensor Systems

Work Unit 056 Diseases of military animals

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McGough, PVT Roy Berriere, PVT Leonard Beller, PVT
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Description.

To define, study, diagnose and control known and potential infectious diseases of military dogs. The major effort is directed toward the cause, pathogenesis, treatment and control of tropical canine pancytopenia (TCP) which jeopardized the operational efficacy of military dogs in SE Asia. Studies are also being conducted on the epidemiology, treatment and control of other viral and parasitic infections which are medical problems in military dogs.

During the reporting period research activities have been concerned with: (1) the clinical hematological and immune response in German Shepherd dogs during acute and chronic Ehrlichia canis infection, following treatment with tetracycline, and challenge inoculation with the homologous strain of Ehrlichia canis; (2) infectivity and pathogenesis of an equine ehrlichial agent in dogs and other animals; (3) characterization of the agent of salmon poisoning disease; (4) use of the monocyte and bone marrow culture techniques for the isolates of Rickettsia rickettsi from experimentally infected guinea pigs; (5) respiratory disease in military dogs; (6) pathogenicity of the canine coronavirus isolate in neonatal dogs and swine; (7) trachea organ cultures. Some of the investigations reported here have been done jointly with Dr. Miodrag Ristic and other investigators at the University of Illinois.

Progress.

1. Clinical and hematologic response of German Shepherd dogs to *Ehrlichia canis* infection, tetracycline therapy, and challenge inoculation.

It is known that experimentally infected German Shepherd dogs often develop a severe hemorrhagic syndrome, but that experimentally infected Beagles do not [1]. Nonetheless, in both breeds TCP is characterized by pancytopenia [1], persistent infection [2,3], hypergammaglobulinemia [4,5], and systemic, primarily perivascular, plasmacytosis [6-8]. These findings have led to speculation that some of the changes seen in TCP may have an immunopathologic basis [5].

Experimentally infected Beagles and German Shepherd dogs develop a similar acute phase disease of 2 to 4 weeks duration characterized by mild clinical signs and a transient pancytopenia [1]. During this period intracytoplasmic inclusions of *E. canis* can be observed in circulating mononuclear leukocytes. In Beagles and some German Shepherd dogs no further clinical illness occurs, but dogs remain infected with *E. canis* and are classified as having non-severe chronic TCP (NCTCP). In contrast, the majority of German Shepherd dogs, 50 to 100 days postinfection (PI), develop severe manifestations of TCP characterized by marked pancytopenia, hemorrhage, peripheral edema, emaciation, and secondary bacterial infections. Most of these dogs succumb due to hemorrhage or secondary infection. These dogs are classified as having severe chronic TCP (SCTCP), the syndrome observed in naturally infected German Shepherd dogs in Southeast Asia [3].

Some question has existed in the past as to whether or not SCTCP is merely a relapse, albeit more severe, from the earlier acute phase disease. We now hypothesize that the changes seen in SCTCP have a different pathogenesis than those seen in acute TCP. To elucidate further the pathogenesis of chronic TCP, we studied German Shepherd dogs with acute and chronic *E. canis* infection, and evaluated the response of dogs with SCTCP and NCTCP to tetracycline therapy. We determined the clinical and hematologic response to tetracycline, the efficacy of tetracycline in clearing dogs of persistent infection with *E. canis*, and the susceptibility of cleared dogs to reinfection.

Fourteen purebred German Shepherd dogs, four males and ten females, between one and two and a half years of age, formed the basic experimental group. Purebred Beagle dogs of both sexes, one to three years of age were also utilized. All dogs were vaccinated according to standard recommendations for canine distemper, infectious canine hepatitis, leptospirosis, and rabies. At time of acquisition dogs were serologically negative for *E. canis* antibodies as determined by the indirect fluorescent antibody test recently reported by Ristic, et al. [9]. Dogs were housed indoors and offered commercial dry food and water free choice.

During critical periods dogs were examined and rectal temperatures were taken one or more times daily; at other times such examinations were performed twice or thrice weekly. Thrice weekly, venous blood was collected in sealed vacuum glass tubes (Vacutainer, Becton-Dickinson, Rutherford, N.J.) with EDTA as anticoagulant. White blood cell (WBC) and red blood cell (RBC) counts were performed on an electronic cell counter (Model B, Coulter Electronics, Inc., Hialeah, Florida). Platelet counts were also performed electronically (MK-4 Platelet Counter, General Sciences Corp., Bridgeport, Conn). Packed cell volumes were determined by standard microhematocrit methods. One hour erythrocyte sedimentation rates (ESR) were determined in Wintrobe tubes. Hemoglobin was determined by the cyanmethemoglobin method. Bone marrow aspirations using light sodium thiamylal anesthesia, were performed on eight dogs in the basic experimental group. Results will be reported elsewhere.

The isolant of E. canis used in this study was originally recovered from a naturally infected German Shepherd dog in Southeast Asia. The organism was maintained by passage in laboratory Beagles. This isolant has been shown to be free of Babesia spp. and Hemobartonella spp. by passage in splenectomized dogs [10]. Initial infection of the 14 dogs in the experimental group was by intravenous inoculation of five ml of whole, heparinized blood (20 units/ml) from a dog acutely ill with TCP. Challenge inoculation of dogs was similarly administered.

Infectivity of blood with E. canis was determined by IV injection of laboratory Beagles with five ml of heparinized blood. Beagles were observed for signs of E. canis infection by thrice weekly hematologic examination for a period of 30 days. Results were confirmed serologically at 30 and 45 days after injection, using the IFA test.

Tetracycline hydrochloride, 250 mg tablets, was administered orally at a dosage of 30 mg/lb/day in two divided doses, for 14 consecutive days. No other antibiotics or supportive treatment was administered to any of the test animals.

Evaluation of hematologic response to treatment was as follows. The course of infection was divided into six time periods for each dog: (1) a control period of 30 days immediately preceding infection with E. canis; (2) a pretreatment period of 14 days immediately preceding onset of outward signs of SCTCP or initiation of treatment; and (3) four periods of 30 days each covering the 120 consecutive days immediately following the initiation of treatment. Mean platelet, WBC, and RBC counts for each animal were then calculated for each time period. When applicable, significance of difference was evaluated using the Student's t test. Level of significance was set at $p < .01$.

Dogs were observed for at least 30 days prior to infection to establish baseline data for each animal. The dogs were infected with E. canis and observed throughout the acute phase illness. Treatment was initiated in SCTCP dogs with outward signs of disease, including hemorrhage. Dogs developing SCTCP with pancytopenia but without outward signs of illness or hemorrhage were arbitrarily placed on tetracycline at 105 days PI. Dogs with NCTCP were observed for 145 days PI and then treated. To determine susceptibility to reinfection, selected, surviving dogs from both SCTCP and NCTCP groups were challenged with the homologous strain of E. canis.

All 14 German Shepherd dogs inoculated with E. canis developed typical acute phase TCP within 10 days PI (Figures 1 and 2). All dogs were febrile, anorectic, and lethargic. Many had a serious conjunctivitis and rhinitis, and manifested loss of weight, lymphadenopathy, diarrhea, and emesis. Pancytopenia was evident by 10 to 14 days PI. No signs of hemorrhage or peripheral edema were apparent at this time, all dogs regained a normal physical appearance, and hematologic values generally improved within 21 to 28 days PI.

Nine of the 14 dogs developed SCTCP (Table 1). The onset of SCTCP was heralded by changes in the hemogram which began 40 to 60 days PI. Severe thrombocytopenia and leukopenia were the earliest and most consistent manifestations of severe disease (Figure 1). SCTCP did not develop in five dogs, classified as having NCTCP and without severe hematologic changes (Figure 2). All NCTCP dogs maintained a normal physical appearance.

Outward signs of illness and hemorrhage occurred in seven of the nine dogs with SCTCP 56 to 98 days PI (Table 1). Depression and weakness were seen in most of these animals, while the occurrence of corneal opacity, emaciation, anorexia, fever, and peripheral edema were variable signs. Treatment was initiated within 12 to 72 hours after the onset of outward signs of SCTCP, with the exception of dog No. 58 which, because of the equivocal nature of the initial hemorrhage, was not treated until seven days after the first signs were observed. Dogs No. 57 and 64 did not manifest outward signs of illness by 105 days PI, but had been severely thrombocytopenic and leukopenic for more than 30 days. Treatment was initiated on these dogs at 105 days PI.

Clinical responses in the nine dogs with severe chronic TCP are summarized (Table 1). Two dogs died during the treatment period, showing no apparent clinical improvement. All dogs with outward signs of illness and hemorrhage that survived the treatment period showed some improvement in physical appearance and activity within seven to fourteen days after the start of treatment (PT). However, varying degrees of depression, emaciation, weakness and fever persisted in most SCTCP dogs for 30 to 60 days. In all dogs with

Table 1. Summary of clinical signs before treatment and clinical response after treatment with tetracycline in nine dogs with severe chronic tropical canine pancytopenia.

Dog no.	Outward signs of severe chronic TCP					Character of signs			Clinical response to tetracycline therapy
	Days outward signs appeared	PI	Depression & weakness	Epi-staxis	Hematomas	Genital pact hemorrhage	Hemorrhage from lacerations	Peri-pherical edema	
54	65	+	+	+	-	-	-	+	Improved; edema and hemorrhage stopped. Worsened 42 days PT and died 49 days PT with pancytopenia.
55	76	+	+	-	+	-	-	+	Developed abscess, bacteremia. Died 13 days PT.
57	105*	-	-	-	-	-	-	-	No changes observed.
58	73	+	+	-	+	-	-	+	Worsened; died with pancytopenia 1 day PT.
60	95	+	+	-	-	-	+	-	Improved; hemorrhage stopped. Developed bacteremia and died 32 days PT.
61	89	+	+	+	+	+	-	-	Improved; hemorrhage stopped. Remained emaciated and weak for months.
64	105*	-	-	-	-	-	-	-	No changes observed.
65	56	-	-	+	+	-	-	-	Improved; hemorrhage stopped. Mild hemorrhage 90 days PT; regained normal appearance.
66	98	+	+	-	-	+	+	-	Improved; hemorrhage stopped. Regained normal appearance.

* Although showing no outward signs of SCTCP these dogs had been pancytopenic for 30 days and were treated empirically at 105 days postinfection. (PI) indicates postinfection. (PT) indicates post treatment.

FIGURE 3.

Summary of hematologic changes in 14 German Shepherd dogs that developed severe chronic TCP (SCTCP) or non-severe chronic TCP (NCTCP). Six time periods are represented: a 30 day preinfection control period, a 14 day period immediately preceding treatment, and four post treatment periods of 30 days each. Each symbol represents the mean cell count during a period for an individual dog. Mean platelet count; SCTCP dogs (●), NCTCP dogs (○). Mean WBC count; SCTCP dogs (■), NCTCP dogs (□). Mean RBC count; SCTCP dogs (▲), NCTCP dogs (△).

external hemorrhage and peripheral edema, remission of these signs occurred by 14 days PT, although one dog had a transient hemorrhagic episode after the completion of treatment. Two dogs died following treatment, dog No. 54 at 49 days PT and dog No. 60 at 32 days PT, while the remaining five dogs survived.

Clinical signs typical of terminal SCTCP were noted in all four dying dogs. Necropsy was performed on three of the dogs. Pathologic diagnosis in all three dogs was terminal SCTCP with massive internal hemorrhages, evidence of secondary bacterial infections, and generalized perivascular plasmacytosis.

The five dogs with NCTCP did not develop outward manifestations of illness and appeared to be normal healthy dogs at 145 days PI. No change in their clinical status occurred following treatment with tetracycline.

Infectivity of blood was determined in all dogs surviving the treatment period. Blood from dog No. 55, which died 13 days PT, was negative for E. canis at the time of death. Blood from dogs No. 54 and 60, which succumbed after the treatment period, was negative at 44 and 32 days PT respectively. Blood from all dogs surviving SCTCP was negative for E. canis when subinoculated at 44 and 104 days PT.

Dogs that developed SCTCP had slightly lower mean preinfection platelet and WBC counts, and slightly higher mean RBC counts than dogs that developed NCTCP, but the differences were not significant ($p > .10$).

During the 14 day pretreatment period all nine dogs with SCTCP had thrombocytopenia (mean \pm SE, $9,800 \pm 1,800/\text{mm}^3$) ($p < .001$), leukopenia (WBC, $3,570 \pm 460/\text{mm}^3$) ($p < .001$), and anemia (RBC, $4.54 \pm .33$ million/ mm^3) ($p < .01$) (Table 1 and Figure 3). As a group, dogs with NCTCP had slightly subnormal pretreatment platelet counts ($183,000 \pm 21,800/\text{mm}^3$) and WBC counts ($10,700 \pm 1,200/\text{mm}^3$), but these differences were not significant ($p > .10$). When NCTCP dogs were evaluated individually however, we found statistically significant thrombocytopenia in three, leukopenia in two, but anemia in none of the five dogs during the pretreatment period.

Pancytopenia persisted in SCTCP dogs in spite of tetracycline therapy (Figures 1 and 3). Dogs surviving SCTCP remained thrombocytopenic and leukopenic for at least 120 days PT, and two remained anemic for that period. Anemia abated in the other three dogs between 30 and 90 days PT. Irrespective of persistent pancytopenia, platelet, WBC, and RBC counts in all five surviving SCTCP dogs gradually improved during the 120 day PT period. Of the four dogs that died with SCTCP, three could be evaluated during or following treatment. No significant hematologic response was seen in dogs No. 55 or 60. Dog No. 54

had a transient improvement in platelet, but not WBC or RBC counts during the zero to 30 day PT period. All three dogs died with severe pancytopenia.

Dogs with NCTCP that had depressed cell counts had variable responses to tetracycline therapy. One of the three dogs with thrombocytopenia 14 days pretreatment had normal platelet counts by 30 days PT, and one of the dogs with pretreatment leukopenia had normal WBC counts by 90 days PT. In dogs that did not respond, thrombocytopenia and leukopenia persisted for at least 90 days PT.

When the hematologic data collected from SCTCP dogs during the 14 day pretreatment period was evaluated it was found that the four dogs dying of SCTCP, as a group, had significantly lower pretreatment WBC counts ($p < .001$) and RBC counts ($p < .01$) than the five surviving SCTCP dogs. There were no significant differences in pretreatment platelet counts between dying and surviving SCTCP dogs ($p > .20$).

At 236 days PI (at least 90 days PT) six surviving treated dogs, three SCTCP and three NCTCP, were inoculated with E. canis. All six inoculated dogs showed some clinical or hematologic signs of acute E. canis infection. Two SCTCP dogs (No. 65 and 66) had characteristic acute phase disease and subsequently developed SCTCP with severe pancytopenia (Figure 1). One dog (No. 66) died with massive intestinal and serosal hemorrhages 36 days post challenge (PC) and was found to have pathologic lesions typical of SCTCP, including generalized plasmacytosis. The second dog (No. 65) suffered a hemorrhagic episode 55 days PC, survived and recovered a normal physical appearance, but was still pancytopenic 90 days PC. Dog No. 64, which originally had SCTCP, and the three NCTCP dogs responded to challenge inoculation with clinical and hematologic changes more mild and of shorter duration than usually encountered in acutely infected German Shepherd dogs. A previously uninfected German Shepherd dog, serving as a positive control and receiving the same challenge inoculum, developed typical acute phase TCP, and subsequently NCTCP. Blood from all six challenged dogs was found to be positive for E. canis. Blood from four of the dogs was infective at 49 days PC. Blood from dogs No. 53 and 63 was not infective at 49, 105, or 149 days PC, but was positive 188 days PC.

In a previous study in this laboratory, Amyx et. al. [10] evaluated the efficacy of tetracycline given to Beagle and German Shepherd dogs at the peak of acute phase experimental TCP. They found that when dogs acutely ill with E. canis were treated with tetracycline defervescence occurred within 24 hours; platelet counts, WBC counts, and ESR returned to normal within seven days; and RBC counts were normal by 14 days. In contrast to the rapid recovery of dogs treated during acute TCP, we found that dogs surviving SCTCP showed very gradual clinical improvement following treatment and that

pancytopenia persisted for months. This difference in response to tetracycline supports the hypothesis that SCTCP has a different pathogenesis than acute TCP.

During acute infection ehrlichiae undergo initial, rapid multiplication, and if the numbers of E. canis inclusions observed in peripheral leukocytes are an indication, it is at this time that the level of parasitemia is the highest. Accordingly the occurrence of fever, clinical illness, and initial hematologic changes suggest an acute infectious process. The transient pancytopenia of acute phase TCP may be similar to the pancytopenia seen in kala-azar, and in some bacterial, rickettsial, and viral infections [11,12]. In these diseases decreased cell counts occur with a normal or hypercellular bone marrow, indicating increased sequestration or destruction of blood cell elements rather than decreased production. That this is the case in acute TCP is suggested by the prompt response of blood cell counts to tetracycline administered during the early phase of illness.

In contrast the persistence of pancytopenia following treatment of our chronically infected dogs appears to be related to impaired production of blood cells in SCTCP. Hildebrandt et. al. [6,7] have suggested that there may be bone marrow hypoplasia in SCTCP. In man, pancytopenia with marrow hypoplasia, or aplastic anemia, may be idiopathic [13,14] or may result from idiosyncratic reactions to drugs such as chloramphenicol or phenylbutazone [13-16], radiation exposure [14], or infections such as viral hepatitis [17,18]. Irregardless of etiology, pancytopenia is often irreversible, patients dying with severe hemorrhage due to thrombocytopenia, or with secondary infections due to leukopenia [13,14]. Additionally, many patients surviving aplastic anemia regain normal hematologic values only after a prolonged course, some having depressed cell counts for years. This is believed to be due to severe depletion of hematopoietic stem cells or irreversible changes in the bone marrow vascular microenvironment, or both [14,19,20,21,22], such changes persisting in the absence of the original causative agent. Persistence of pancytopenia in SCTCP following apparent removal of the etiologic agent, and the terminal course followed by some dogs in spite of treatment suggests that aplastic anemia may be of major importance in the pathogenesis of SCTCP.

That we were unable to recover infectious E. canis from the blood of any dog receiving the full 14 days course of tetracycline is presumptive evidence that tetracycline is effective in clearing dogs of persistent infection. Nonetheless, it is possible that dogs remained infected and that (1) the numbers of circulating infectious units of E. canis were below levels likely to be detected by subinoculation; or (2) viable organisms were harbored in host tissues other than the blood, as is believed to occur in another rickettsial disease, recrudescent typhus [23]. However, the fact that all six previously

treated dogs challenged with the homologous strain of E. canis had some signs of infection and became carriers of the organism provides further evidence that dogs were completely freed of the original infection, following tetracycline therapy. This is of particular importance since chronically infected dogs may serve as a source of infection for other dogs via the tick vector.

Results of challenge inoculation of previously infected, cleared dogs revealed that although two challenged dogs developed clinical and hematologic changes typical of acute phase TCP, in four dogs the hematologic and clinical responses to challenge were remarkably mild. Leeflang [24] found a somewhat similar response after he challenged three dogs cleared of chronic E. canis infection by oxytetracycline. His dogs did not develop clinical or hematologic signs of infection post challenge, but did become carriers of the organism. In contrast, Amyx, et al [10] reported that all dogs treated during initial acute phase TCP developed typical acute phase disease when subsequently reinoculated 90 days later. Apparently, chronic infection of some dogs with Ehrlichia canis confers increased resistance to reinfection.

2. Response of serum antibody and gammaglobulin in German Shepherd dogs to Ehrlichia canis infection, tetracycline therapy, and challenge inoculation.

Dogs infected with Ehrlichia canis, the etiologic agent of tropical canine pancytopenia (TCP) remain carriers of the organism indefinitely. Such persistent infection occurs irrespective of the development of specific humoral antibodies, hypergammaglobulinemia, and systemic proliferation of plasma cells. Similar developments occur in Aleutian mink disease, which is considered to be an example of an immuno-pathologic disease [25,26]. An immunologic pathogenesis of disease has been suggested for TCP [5,9].

In the preceding study the clinical and hematologic responses of 14 German Shepherd dogs were evaluated following Ehrlichia canis infection, tetracycline therapy, and challenge inoculation. Following an acute phase illness in all dogs, nine developed severe chronic TCP (SCTCP) characterized by severe pancytopenia, hemorrhage, and secondary bacterial infection, while five had non-severe chronic TCP (NCTCP) with only mild pancytopenia. In spite of tetracycline therapy, four SCTCP dogs died while five survived. Hematologic values in surviving SCTCP dogs improved very gradually, thrombocytopenia and leukopenia persisting for at least 120 days post treatment. NCTCP dogs showed only minor hematologic changes following treatment. E. canis could not be recovered from the blood of any treated dog. Six surviving, treated dogs, when challenged with the homologous strain of E. canis, became reinfected and two developed severe disease.

This study afforded an opportunity to define the immunologic response in German Shepherd dogs during acute and chronic E. canis infection, following treatment with tetracycline, and challenge inoculation with the homologous strain of E. canis.

Detailed serial studies of serum antibodies and gammaglobulin had not been previously undertaken in German Shepherd dogs, which often develop a severe and fatal illness during chronic infection with E. canis.

The experimental design and procedures are given in the preceding study. Blood for serological tests was collected weekly. Sera were stored at -20 C until time of test. Serum antibodies to E. canis were determined by the use of indirect fluorescent antibody (IFA) test recently described by Ristic et al. [9]. All serial specimens were tested simultaneously. Sera were screened at a dilution of 1:10; positive samples were serially diluted 2-fold and titrated.

Protein of selected serum samples from nine dogs in the experimental group were separated by electrophoresis, using cellulose acetate strips (Tital III, Helena Laboratories, Beaumont, Texas) and a Tris-barbital-sodium-barbital buffer. Fractionated protein was stained with Ponceau S. (Helena Laboratories), scanned with a Quick-Scan densitometer, and quantitated with a Quick-Quant digital computer (both from Helena Laboratories). Total protein was determined using a TS refractometer. Serum gammaglobulin was expressed as percent of total serum protein.

All dogs were seronegative for E. canis antibodies prior to infection. The earliest positive conversion occurred seven days postinfection (PI) in two dogs, and all but one dog were seropositive by 20 days PI (Table 2). Titers in all dogs increased from seroconversion to a maximum titer at 42 to 98 days PI. Evaluation of the geometric mean titer (GMT) of all dogs before treatment revealed that GMT continued to increase until approximately 80 days PI (Figure 4A).

Following tetracycline treatment titers decreased from a pretreatment GMT of 1:110 to 1:56 at 100 days post treatment (PT), (Figure 4B). Individual titers during the 81 to 100 day PT period ranged from 20 to 160. Titers in four treated dogs that could be evaluated for an additional 100 days PT remained essentially unchanged during this period.

Following challenge inoculation of six treated dogs with the homologous strain of E. canis, two-fold or more increases in IFA titer occurred in four dogs by day six and in one dog by day 14 post challenge (PC), (Figure 4C). In one dog the IFA titer had increased by 33 days PC; this dog developed SCTCP and died 36 days PC. The GMT for both SCTCP and NCTCP dogs approximately doubled

Table 2. Time of seroconversion in fourteen German Shepherd dogs infected with Ehrlichia canis.

Days postinfection	Number of dogs seronegative	Number of dogs seropositive
Preinfection	14	0
7	12	2
13	5	9
20	1	13
26	1	13
42	0	14

by 14 days PC. Between 21 and 33 days PC, the GMT for the three SCTCP dogs continued to increase, whereas titers in all NCTCP dogs dropped to the prechallenge level.

Changes in serum protein were serially evaluated in four SCTCP and two NCTCP dogs. Moderate hypoproteinemia occurred in five of the six dogs between 20 and 42 days PI, but by 63 days PI values for total protein were normal. Decreases in percent albumin occurred in five dogs by 20 to 26 days PI, and in the remaining dog by 42 days PI. The magnitude of these changes were similar to those reported by Burghen et al. [5]. No significant change occurred in alpha¹ globulin, but four of six dogs had moderate decreases in percent alpha² globulin and increases in beta globulin during the acute phase of infection.

All six dogs were hypergammaglobulinemic by PI day 20 (Figure 5A). Gammaglobulin levels increased two-fold from a preinfection mean (\pm SE) of 10.5 ± 0.72 to 21.7 ± 3.03 percent at 26 days PI. Hypergammaglobulinemia persisted in all but one dog until treatment was initiated (Figures 5A and 5B). In one NCTCP dog, percent gammaglobulin had dropped to normal by 123 days PI, before treatment.

Following tetracycline therapy, gammaglobulin levels in all five dogs with hypergammaglobulinemia decreased to preinfection values, although this occurred faster in some dogs than in others (Figure 5B). The most rapid decreases in gammaglobulin were observed in the two dogs that died with SCTCP after the treatment period.

Four dogs were evaluated for changes in serum gammaglobulin following challenge inoculation with *E. canis*. Percent gammaglobulin increased from a prechallenge mean (\pm SE) of 10.8 ± 1.4 to 26.3 ± 3.6 at 21 days PC (Figure 5C). However, hypergammaglobulinemia persisted in only two of the four challenged dogs; two NCTCP dogs had normal gammaglobulin levels by 97 days PC.

It is clear that a humoral antibody response occurs in dogs infected with *E. canis* and that this response does not prevent persistent infection. The continued increase in antibody titer for 80 days PI was consistent with the development of hypergammaglobulinemia, and marked plasmacytosis [1,27]. This apparent excessive production of immunoglobulins occurs similarly in several viral diseases which are characterized by persistent infection [25,26,28,29,30].

IFA titers persist following termination of infection with tetracycline; however gammaglobulin levels, which are elevated during the carrier state return to normal when infection is terminated. Correlation between gammaglobulin level and infectivity may have importance in the clinical diagnosis of TCP. Gammaglobulin levels may be useful as a diagnostic aid in differentiating carrier dogs from dogs which have been cleared of the infection.

The antibody response, hypergammaglobulinemia, and plasmacytosis in dogs experimentally infected with TCP occurred after acute phase illness and peak parasitemia have passed. Chronic phase illness (SCTCP) did not become apparent for at least 40 to 60 days PI, when serum antibody and gammaglobulin are markedly elevated. This suggests that the immune response may be a factor in development of SCTCP.

As would be expected, dogs challenged with *E. canis* manifested an anamnestic antibody response with a two-fold increase in titer within six days. The magnitude of increases in gammaglobulin were greater following the challenge infection than in the original infection, but whether the increase occurred sooner is not clear.

Differences in serum antibody response to challenge in SCTCP and NCTCP dogs, as well as in the postchallenge clinical and hematologic responses of these two groups serve to suggest, within the limitations of sample size, a resistance to *E. canis* in NCTCP dogs, but not all SCTCP dogs. This resistance, however, was not reflected in the prechallenge IFA titers. SCTCP dogs reacted to challenge infection almost as if they had not previously been exposed to *E. canis*. This may indicate some degree of immunologic unresponsiveness in SCTCP dogs, again not reflected in IFA antibody changes.

Antibody and gammaglobulin changes occurring in TCP are similar to those in Aleutian mink disease where gammaglobulin levels may increase three to four-fold by 30 days PI, and IFA titers reach 100,000 or more [25,26]. Antibody titers in TCP do continue to increase well into the chronic phase of infection, as occurs in Aleutian mink disease, and severe disease in both TCP and Aleutian mink disease usually occurs 2 months or more after infection [25,27]. Further study of immunologic changes in TCP may reveal further similarity with Aleutian mink disease. Unlike the situation with the viral induced immunopathologic diseases, infection with *E. canis* can be readily terminated, facilitating study of the host-parasite interaction.

3. Studies on the infectivity and pathogenesis of an equine ehrlichial agent in dogs and other animals.

Equine ehrlichiosis has been reported since 1969 in horses originating from the foothills of the Sacramento valley in California [31]. The disease is characterized by low mortality, fever, anorexia, depression, edema of the legs, ataxia, leukopenia, thrombocytopenia, mild anemia, and inclusions in the cytoplasm of neutrophils and eosinophils. Sheep, goats, and dogs have been shown to be susceptible to experimental infection with the agent but the clinical signs of infection in these animals were mild or inapparent. The inclusions in the cytoplasm of neutrophils and eosinophils of infected animals were morphologically similar to those previously described for ehrlichia or ehrlichia-like agents of cattle, sheep and dogs.

Ehrlichia canis, a tickborne rickettsial agent of world wide distribution, has been reported to produce a relatively mild disease except in puppies or debilitated older dogs. However, recent reports of tropical canine pancytopenia (TCP) have shown that the severity of disease caused by Ehrlichia canis is dependent upon the breed of dog. The severe disease produced by E. canis in the German Shepherd dog prompted a study of the equine ehrlichial agent. The objectives of the study were to (1) confirm the infectivity of the equine ehrlichial agent for dogs; (2) examine the pathogenicity of the equine ehrlichia in both German Shepherd and Beagle dogs; (3) study the serologic relationship of the equine ehrlichial agent and Ehrlichia canis; and (4) determine the host range of the equine ehrlichial agent.

Whole blood from infected horses was obtained in the frozen state from Dr. David Gribble, University of California. The blood was thawed and used to inoculate two horses. During the acute stage of infection when morulae were evident in 15 to 30% of the circulating granulocytes blood was collected in EDTA and used to inoculate other animals. Infected blood was also preserved in liquid nitrogen for further passage. The isolant of E. canis used in this study was recovered from a German Shepherd dog with typical signs of TCP in Southeast Asia.

Twenty-five Beagle and German Shepherd dogs, 14-26 weeks of age were used in the study. The dogs were previously vaccinated for distemper, hepatitis, and leptospirosis according to standard recommendations. The Beagles were inoculated intravenously with 3 ml and the German Shepherds with 5 ml of infected blood. In addition some of the dogs were also inoculated intraperitoneally with 5 ml of infected blood.

Five domestic short hair cats, four females and one male, each three months of age, and vaccinated for feline panleukopenia, were inoculated intravenously with 3 ml of infected blood containing the equine agent.

Twelve Rhesus monkeys, 4 to 6 lbs each, and two baboons, 7 and 18 months of age, were inoculated. Rhesus monkeys were of both sexes. Both baboons were males. All primates were inoculated intravenously with 2 ml of infected horse blood. Six of the primates were also inoculated subcutaneously with 5 ml of infected blood and six with 5 ml intraperitoneally.

Eighteen white mice strain WRAIR ICR, 7 to 9 weeks of age; 20 WRC rats 75 to 85 gm each; 20 young adult golden hamsters; 20, 200-250 gm Hartley strain guinea pigs; and six, 3-4 lb New Zealand white rabbits were inoculated by a combination of intravenous, intraperitoneal and subcutaneous routes.

Eight mice were inoculated intravenously with .05 ml of fresh infected horse blood; five received .25 ml intraperitoneally and five .25 ml subcutaneously. Ten rats, 10 hamsters and 10 guinea pigs were each

inoculated intraperitoneally with 1 ml of infected horse blood. Similar groups of 10 rats, hamsters, and guinea pigs were each injected with 1 ml of the horse blood subcutaneously. Each of the rabbits was also inoculated with 1 ml of infected horse blood, three rabbits intravenously and three subcutaneously.

Dogs, cats and primates were examined and rectal temperatures recorded daily. The primates were sedated with ketamine hydrochloride before rectal temperatures were recorded. Blood was collected from each of these animals daily and examined to determine if morulae were present in circulating granulocytic cells. Two and often three times a week, depending upon size and species, blood was collected in sealed vacuum tubes containing EDTA anti-coagulant for clinical laboratory examination. Every two days blood was collected from each of the small laboratory animals and a buffy coat preparation from the blood was examined for morulae. All laboratory tests were conducted within 2 hours after specimens were collected. White and red blood cell counts were determined with an electronic cell counter. Thrombocyte counts were determined with an electronic platelet counter. A standard microhematocrit centrifuge was used for all packed cell volume determinations. Hemoglobin determinations were made by the cyanmethemoglobin method. The Wintrobe tube was used for determination of erythrocyte sedimentation rates.

Buffy coat smears were prepared from peripheral blood and stained with either Giemsa or Wright's stain. A minimum of 300 neutrophils were examined under oil immersion (1,000 x) in each preparation. On a few occasions this number of cells was difficult to obtain without the preparation of several buffy coat smears. An animal was considered to be infected with the equine agent when two or more neutrophils, or eosinophils, containing blue-gray mulberry shaped structures composed of loose or compact spheres, or short rounded rods, were observed. These structures were within the cytoplasm of the encompassing granulocyte.

A complete necropsy was performed on each dog, primate and cat. Tissues were collected and fixed in 10% formalin and processed for histopathology examination by routine methods. The morulae observed in the dogs, horses, and cats were indistinguishable. Within the granulocytes of each individual animal, the size and shape of the structures composing the morulae, as well as the morulae themselves, varied greatly. These morulae ranged in diameter from 1.5 to 5.0 μ . Morulae were usually one to a cell, but occasionally two and even three were noted in the same neutrophil. Morulae were observed in the eosinophils of cats, neutrophils of primates and in both neutrophils and eosinophils of horses and dogs.

Many other cytoplasmic inclusions, which varied in density, color, size and shape, were noted a day or two prior to the appearance of morulae. Many of these same structures were also observed when morulae were

present. Neutrophils containing cytoplasmic inclusions other than morulae were observed in dogs, nonhuman primates, guinea pigs and mice. These inclusions consisted of a single, or often a pair of dark blue-black sphere(s) approximately $.2\ \mu$ in diameter. These inclusions were not considered as evidence of infection. Gross or microscopic lesions attributable to the infection could not be demonstrated in any of the animals or tissues examined.

None of the 25 dogs inoculated with the equine ehrlichial agent demonstrated any of the outward clinical signs of anorexia, conjunctivitis, or hemorrhage, typical of German Shepherd dogs infected with Ehrlichia canis. Morulae were observed in the circulating granulocytes of 15 of 25 dogs from 7 to 22 days after inoculation. The presence of morulae coincided with a mild pyrexia, a short transient thrombocytopenia and decreased hematocrit. Morulae appeared in most of the dogs between the 7th and 16th day after inoculation, and persisted for 1 to 4 days. Only a few of the dogs became leukopenic and/or demonstrated an increased sedimentation rate. Hematologic values of all 25 dogs returned to preinfection levels within 2 weeks following the disappearance of morulae. Morulae were observed in 12 of the inoculated Beagles and 3 of 8 German Shepherds. Two to 4 months after the disappearance of morulae, 8 of these dogs, 5 Beagles and 3 German Shepherds, were challenged with E. canis infected blood. Within 2 weeks after inoculation all eight of the challenged dogs developed the typical signs of tropical canine pancytopenia, exclusive of epistaxis. The signs included severe pyrexia, anorexia, greatly increased erythrocyte sedimentation rate, a severe thrombocytopenia, anemia and leukopenia.

Morulae were observed in eosinophils of 1 of 3 cats inoculated with infected horse blood and 1 of 2 cats inoculated with infected dog blood. In none of the cats were morulae seen in neutrophils. The cats were only mildly affected. Eosinophils containing morulae were present on days 7 and 8 after inoculation. A definite eosinophilia occurred in both cats during the period morulae were observed.

Of the 14 inoculated nonhuman primates morulae were observed in the blood of 7 Rhesus monkeys and 1 baboon. No abnormalities in habits, appetite or physical condition were noted in any of the inoculated animals. Morulae were noted only in the mature neutrophils of the primates. As in some dogs single small blue-black spheres were seen in the cytoplasm of neutrophils of several of the infected primates. Morulae were present within 3 to 7 days after inoculation. Blood collected from two of the infected Rhesus monkeys was used to successfully infect a second horse. Infected blood collected from this second horse during the acute stage of illness was injected into 4 susceptible Rhesus monkeys. These 4 monkeys were closely observed and temperatures recorded twice daily. A small quantity of blood was collected every 12 hours from the monkeys for buffy coat preparations. Morulae first appeared in the neutrophils of one of the

monkeys on the 3rd day after inoculation and continued to be present through the 6th day. Two of the remaining 3 monkeys demonstrated morulae on the 5th day. In one monkey morulae were also detected on the 6th and 7th day following inoculation. Morulae were not observed in the granulocytes of the 4th monkey.

The 3 Rhesus monkeys in which morulae were observed experienced a definite pyrexia, increased erythrocyte sedimentation rate, a mild thrombocytopenia and leukopenia, as well as a mild anemia. All animals returned to normal within one week of the disappearance of morulae.

Granulocytes containing morulae were not observed in any of the rats, mice, guinea pigs, hamsters and rabbits inoculated with the agent. However neutrophils containing cytoplasmic inclusions were noted in several of the guinea pigs and mice. These inclusions did not meet the criteria for morulae but many were of identical color, size and shape as the dark blue-black spheres, appearing singularly, in pairs or as triads.

None of the small laboratory animals died of causes attributed to inoculation with the equine ehrlichia. All of the animals continued to grow and gain weight throughout the study.

Beagle and German Shepherd dogs inoculated with the equine ehrlichial agent demonstrated only a mild hematological response and no observable signs of illness. However morulae were present in 12 of 17 Beagles and 3 of 8 German Shepherds inoculated with infected blood. There has been a reported difference in severity of disease in the Beagle and the German Shepherd dogs experimentally infected with E. canis. In the German Shepherd E. canis produces a severe highly fatal, hemorrhagic disease. No differences were noted between the two breeds of dogs infected with the equine ehrlichial agent.

Cytoplasmic inclusions which did not meet the criteria for morulae were present in the neutrophils of primates, some of the small laboratory animals, and dogs. These inclusions appeared to be indistinguishable from those described by Gribble, in mice and guinea pigs, of approximately 0.2 μ in diameter. Such spheres may represent an early stage in the development of the organism within the cytoplasm. These forms at times appeared to be dividing while others seemed to be merely remains of previous morulae and yet other forms were highly suggestive of the life cycle described for some of the chlamydiae. Similar structures were also described by Donatien and Lestoquard as a part of the developmental cycle of E. canis.

Recently Ewing [32] reported the identification of a "new strain" of Ehrlichia canis in a dog in Arkansas. Morulae were found in neutrophils and in the eosinophils of 2 dogs experimentally infected with the "new strain" of Ehrlichia canis. This strain produced relatively mild disease in the naturally infected dog from which the isolant was recovered and in the 2 experimentally infected dogs. The mild nature of disease produced in these dogs is not unlike the mild response reported in this study for the dogs infected with the equine ehrlichial agent. Both agents were characterized by the presence of morulae in granulocytes. From these observations one may speculate that the isolate recently recovered by Ewing and the equine ehrlichial agent may be identical. In addition these agents possess striking morphologic resemblance to the agents of tick-borne fever and bovine petechial fever. A recent report [33] on the ultrastructure of the agent of bovine petechial fever strongly supports the grouping together of the California ehrlichial agent, the agents of tick-borne fever and the agent of bovine petechial fever into a single genus.

The response of dogs infected with the equine ehrlichial agent was dramatically different from the severe disease reported in Beagle and German Shepherd dogs exposed to the isolants of Ehrlichia canis.

Previous infection with the equine ehrlichial agent does not provide protection against subsequent infection with Ehrlichia canis. Each of the 8 challenged dogs, 5 German Shepherds and 3 Beagles, developed signs of TCP (pyrexia, depression, lowered thrombocyte count, elevation of the erythrocyte sedimentation rate, and lowered red and white cell counts) within 7 to 10 days after inoculation with Ehrlichia canis. Morulae of E. canis were observed in lymphocytes and monocytes of both breeds of dogs.

Our successful experimental infection of a variety of previously reported species plus the infection of 3 new species, cat, Rhesus monkey and baboon, tend to further distinguish this agent from the other rickettsial agents of animals. The equine ehrlichial agent was successfully passed from a horse to a group of nonhuman primates, back to a second horse and from this second horse to a second group of nonhuman primates. The agent was identified in the neutrophils of animals from each group. These studies serve to direct attention to the potential transmission of ehrlichial agents to man.

4. Characterization of the agent of Salmon Poisoning Disease (SPD).

A previous report described the in vitro isolation of the SPD agent by using the method described by Nyindo et al., for in vitro cultivation of E. canis. Upon initial isolation the SPD agent was capable of producing an acute lethal infection when inoculated intravenously into Beagle dogs. Continuous in vitro passage of the agent was

possible, and at higher passage the isolate became less virulent for dogs. At the 18th passage the SPD isolate was infectious but non-lethal for dogs. Clinical signs produced by the non-lethal agent were compatible with those reported for Elokomin Fluke Fever.

Challenge studies in dogs using the lethal and non-lethal isolates are in progress. Results indicate that treated dogs which have recovered from infection with the lethal isolate are immune to infection by the non-lethal isolate. However, dogs which have recovered from an infection with the non-lethal isolate are not immune to infection by the lethal isolate.

Using a commercially available anti-canine conjugate it was possible to specifically stain both the lethal and non-lethal isolate in monolayers of canine monocytes by an indirect fluorescent antibody method. Preliminary results of indirect fluorescent antibody staining indicate the lethal and non-lethal isolates are related antigenically.

Initial results based upon morphology, CPE, and indirect fluorescent antibody staining indicate the non-lethal SPD isolate will multiply in primary canine kidney cell cultures. Additional studies are planned to more definitively characterize the SPD agent and to compare disease produced in dogs by SPD and E. canis.

5. Isolation of Rickettsia rickettsi from experimentally infected guinea pigs by primary cultivation of bone marrow cells and circulating monocytes.

The cultivation in primary monocyte cell culture of two rickettsia-like agents of the dog, Ehrlichia canis and Neorickettsia helminthoeca, was recently reported [34,35]. Additionally, E. canis has been isolated from infected dogs using primary bone marrow cell cultures. These findings prompted evaluation of the bone marrow cell and peripheral monocyte culture techniques for the primary isolation of rickettsial agents of man. Initial studies were carried out with Rickettsia rickettsi.

Male Hartley guinea pigs, 400-500 gm, were inoculated intraperitoneally with $1-4 \times 10^3$ fifty percent egg infective doses of a yolk sac suspension of R. rickettsi (Sheila Smith strain). Rectal temperatures were recorded daily. Three to seven days post inoculation (PI) selected animals were anesthetized; blood was collected from the heart and heparinized (20 units/ml), and both femurs were removed aseptically. Blood was centrifuged in 10 ml buffy coat centrifuge tubes (Vir Tis, Arthur H. Thomas Co., Philadelphia) at $500 \times g$ for 10-20 minutes at 4 C. The buffy coat was aspirated, resuspended in autologous plasma, and placed in Leighton-type tissue culture tubes with coverslips. Cultures were incubated at 34-35 C for 18 hours,

washed twice with Hank's balanced salt solution, and fed with tissue culture medium (TCM) (Eagle's minimum essential medium with Earl's balanced salt solution, supplemented with 30-40% guinea pig serum and 0.1 mM L-glutamine per milliliter). TCM was changed every 48-72 hours. Femoral marrow cavities were flushed with TCM and the bone marrow cells were suspended in the TCM by vigorous pipetting. The bone marrow cell suspension was placed into Leighton-type tubes and then handled identically to cultured circulating monocytes. Cultures from uninoculated guinea pigs were also prepared.

At various intervals, 3-11 days after initiation of cultures, coverslips were removed and stained by the Gimenez technique, or with fluorescein conjugated anti R. rickettsi rabbit serum. Conjugated antisera to Rickettsia tsutsugamushi and conjugated normal rabbit serum were used as controls.

Results of two experiments are summarized (Tables 3 and 4). Rickettsiae were isolated in both bone marrow cell and circulating monocyte cultures derived 3 to 7 days PI. Morphologically-typical rickettsiae were readily recognized in Gimenez stained cultures. The number of cultured cells infected with rickettsiae, and the number of organisms per cell increased with duration of culture. Organisms were seen in the nucleus as well as the cytoplasm of infected cells. Rickettsiae in cell culture were specifically stained with fluorescein conjugated R. rickettsi antiserum but not with conjugates of R. tsutsugamushi antiserum or normal rabbit serum. Guinea pigs inoculated with TCM from infected cultures developed typical signs of R. rickettsi infection. Although antibiotics were not used in any cultures, bacterial contamination was not a problem. R. rickettsi was not observed in cultures from uninfected guinea pigs.

Studies are in progress to evaluate the sensitivity of the bone marrow cell culture and monocyte culture techniques and to relate success of culture to levels of rickettsemia, clinical signs, and development of specific serum antibodies. That rickettsiae were recognized as early as 3 days after cultures were initiated suggests the possible usefulness of primary cultures as a means of rapid and specific diagnosis in R. rickettsi infections. It is hoped that these techniques will be applicable to other rickettsial infections as well.

6. Respiratory disease in military dogs.

In May-June of 1966, epizootics of respiratory disease occurred at the military dog procurement and training centers [36,37]. Signs of upper respiratory disease occurred in approximately 33 percent of the dogs at the Ft. Benning scout dog training center. The epizootic disrupted the training and deployment of the dogs. The etiological agent was shown to be parainfluenza SV-5 which was recovered from affected dogs.

Table 3. Isolation of Rickettsia rickettsi from guinea pigs by primary cultivation of bone marrow cells.

Exp. No.	Guinea pig No.	Day post-infection	Days of culture ^a				
			3	4	5	7	10
1	2481	4	ND	-	-	-	ND
	2482	5	ND	+	+	+	+
	2483	6	-	-	+	+	+
	2484	7	+	+	+	ND	+
2	2491	3	-	ND	-	-	ND
	2492	4	-	ND	+	+	ND
	2493	5	-	ND	+	+	ND
	2494	7	-	ND	-	+	ND

^a Plus (+) indicates R. rickettsi observed in culture; minus (-) indicates R. rickettsi not observed; ND indicates not done.

Table 4. Isolation of Rickettsia rickettsi from guinea pigs by primary cultivation of circulating monocytes.

Guinea pig no.	Day post- infection	Days of culture ^a			
		3	5	7	11
2491	3	-	-	-	+
2492	4	-	-	+	ND
2493	5	-	+	ND	ND
2494	7	-	-	+	ND

^a Plus (+) indicates R. rickettsi observed in culture: minus (-) indicates R. rickettsi not observed; ND indicates not done.

By the use of serological tests, the virus was shown to infect almost all the dogs [36,37]. In 1967 and 1968 respiratory disease continued to appear in military dogs at the procurement and training centers [38]. Sero-epidemiological studies indicated that SV-5 infections continued and dogs were infected soon after arrival at the procurement center [38]. Since 1969 the incidence of respiratory disease and SV-5 infections have declined to a very low level. However, more than 85% of newly procured dogs continue to be susceptible to SV-5 infection on the basis of serological test findings (Annual Report 1971-72). This report summarizes further serological studies on SV-5 infections in military dogs at the procurement and training centers.

During the past year paired serum samples were obtained from 203 military dogs upon arrival and departure from the procurement center. One hundred seventy-eight (87%) were devoid of parainfluenza SV-5 antibody. The serologically positive dogs came from 10 states, representing all areas of the United States. None of the serologically-negative dogs developed SV-5 antibody during their stay at the procurement center, indicating infections with this virus did not occur at this time. The 87% incidence of SV-5 serotest susceptible dogs observed this year was essentially the same as those seen in the previous three years.

Tests for SV-5 antibody also were done on serum specimens from 54 dogs at Ft. Benning, Georgia. The dogs were bled in April-May 1973. Forty-one (76%) dogs were serotest positive. Previous test for SV-5 antibody had been done on 19 of these dogs and only 4 were serotest positive. Ten of the remaining 15 dogs converted to positive antibody status. The earlier specimens from the converting dogs were obtained in 1969 and early 1970. Four of the 5 dogs which did not convert were bled in late 1970 and 1971. The time of these SV-5 infections is not known. Infections probably occurred in 1969 and early 1970. All 8 dogs bred at Biosensor Unit at Edgewood were serotest negative. Past sero-surveys of these dogs indicated the absence of SV-5 infections.

7. Pathogenicity of the canine coronavirus isolate in neonatal dogs and swine.

The recovery of 2 canine coronavirus isolates, L198R and 1-71, have been previously described (Annual Report 1971-1972). The L198R isolate was recovered from a laboratory dog with fatal respiratory disease and the 1-71 virus was isolated from military dogs in Germany with diarrheal disease. These viruses were found to be antigenically related but neither were serologically identical to transmissible gastroenteritis virus of swine (TGE). The TGE virus is known to infect dogs [39]. Initial attempts to infect pigs with the L198R virus were unsuccessful (Annual Report 1971-72). The present report summarizes studies on the pathogenicity of the second dog isolate 1-71 for pigs and neonatal dogs.

Pigs free of TGE, 1-71 and L198R neutralizing antibody were obtained from the WRAIR farm at Ft. Meade. In the first experiment 2 1/2 month-old pigs were given approximately 25,000 TCID₅₀ of 1-71 virus by either the intravenous (2 pigs) or combined oral-nasal (4 pigs) routes. No signs of disease were observed post-inoculation. Following inoculation the virus could not be recovered from rectal swab specimens over a 10 day period; neither could specific neutralizing antibody be detected through day 35. When pigs were autopsied no gross or histopathological lesions of TGE were seen. In the second experiment, six 5-day-old pigs were fed approximately 250,000 TCID₅₀ of 1-71 virus. Again clinical signs of disease were not observed. The virus could not be recovered from either throat or rectal swab specimens through 12 days post exposure and there was no detectable antibody to 1-71, TGE or L198R on day 19. At this time the 3-week-old pigs were challenged with approximately 10,000 pig ID₅₀ of virulent TGE. On days 3, 4 and 5 all of the pigs had acute gastroenteritis, manifested by vomiting, diarrhea, weight loss and anorexia. One of the pigs died on the 6th day. Two other pigs were killed for histopathological study. One of these had histological lesions compatible with TGE infection. The other baby pig had no signs of TGE infection. The remaining pigs began to improve on day 7 and all subsequently recovered. On day 20 post TGE challenge each pig had developed TGE neutralizing antibody, titers ranging from 1:2 to 1:32, but no antibody to the canine coronaviruses isolates 1-71 and L198R were found.

The response of neonatal dogs to 1-71 infection were studied in two litters. Three or five days after birth all but 1 or 2 pups in each litter were fed 1-71 virus. Pups in the two litters (G25 and C80) were given 30,000 TCID₅₀ and 500,000 TCID₅₀ of virus respectively. The remaining pups were kept as contact controls. Three to five days after infection the pups of bitch G25 had clinical signs of acute gastroenteritis and dehydration. These signs persisted for 1 to 6 days (Table 5). Concurrent with disease signs, virus was recovered from fecal swab specimens. The virus was usually recovered over a 7 to 8 day period. Neutralizing antibody to 1-71 virus was present 21 days post-infection. Antibody titers to TGE and L198R were often 4-fold lower than 1-71 virus. The response of the second litter (C80) was essentially similar to that of G25 except the clinical signs were milder and the antibody titers to 1-71 and TGE were similar.

The experiments indicate that 1-71 virus is not infectious for pigs but infects neonatal dogs. Mild signs of gastroenteritis were seen in the puppies. This virus may be an important cause of canine diarrheal disease. Further studies are required to define the precise antigenic relationships of 1-71, L198R and TGE.

Table 5. Observations of newborn puppies infected with Canine Coronavirus Isolate 1-71.

Age	Pup No.	Route of Infection	Diarrhea on Days	Virus Recovery on Day		Neutralizing Antibody Titer on Day											
				Throat	Rectum	1-71		TGE		L198R							
						21-28-42-60	21-28-42-60	21-28-42-60	21-28-42-60								
G25 (3d)	1	contact	4-5	9	5-9,12	2	2	8	8	0*	0	0	2	0	0	2	2
	2	oral	4-9	0	5-10,13	2	8	8	8	2	0	8	2	0	0	0	0
	3	oral	5-9	9	3-5,7-10	2	2	2	32	0	0	0	2	0	0	0	0
	4	oral	3-7	0	4-7,9,10,12	2	2	8	8	0	0	2	2	0	0	2	2
C80 (5d)	2	oral	5-9,12	0	5-7,9-11	2	ND**	8	32	2	8	8	ND	0	0	0	ND
	3	oral	6,7,9,10	0	7-12	2	ND	8	8	2	32	8	ND	0	0	0	ND
	4	oral	7	0	6-8,10,13,14	2	ND	8	8	2	8	2	ND	0	0	2	ND
	6	oral	7-9,14	0	5-9,11,12	2	ND	32	8	2	8	8	ND	0	0	2	ND
	1	contact	6,12	0	11-19	2	ND	8	8	0	8	8	ND	0	2	0	ND
	5	contact	7	0	9,11-14,16	0	ND	8	8	0	2	2	ND	0	ND	0	ND

* less than 1:2

** ND = not done

8. Recovery of Reovirus Type II and the Toronto A26/61 Canine Adenovirus from a puppy with respiratory disease.

Reoviruses have been found to be widely distributed in mammalian species. To date only Reovirus Type I has been recovered from dogs with respiratory disease [40,41]. However, serologic evidence of Type II infection has been reported [42]. The present study substantiates the serologic evidence of Reovirus Type II infection by the recovery of this virus from a puppy in the initial stages of respiratory disease. Subsequently a Toronto A26/61 Canine Adenovirus was recovered from several post-mortem tissues.

An unvaccinated puppy (Z-4), purchased for cell culture production, developed a cough and nasal discharge. The signs of respiratory disease continued another 2 months when the puppy developed bronchio-pneumonia and gastroenteritis. The animal was killed and examined. Gross lesions were evident in the lung and small intestine. A few yellowish consolidated areas were seen in the lungs. The small intestine was focally hyperemic and a few ascarid nematodes were in the lumen.

A severe, diffuse, subacute interstitial pneumonia characterized by thickening of alveolar walls, by mononuclear cell infiltration and hypertrophy of alveolar septal cells were revealed by histopathologic examination. Scattered throughout the lung were multifocal areas of polymorphonuclear cell infiltrate in terminal air passages and alveolar spaces. This latter finding coupled with a subacute tracheitis in this animal are suggestive of a primary viral respiratory infection with secondary bacterial insult. A mild, multifocal, nonsuppurative meningoencephalitis was observed in the posterior portion of the brain, i.e., cerebellar peduncles, pons and fourth ventricle. The exact cause of this lesion is unknown. A catarrhal enteritis caused by *Coccidia* sp. was present in the small intestine. Specimens for virus isolation were obtained from nares, throat and rectum two days after disease onset. In addition, specimens from the respiratory tract and other tissues were obtained during post-mortem examination. Blood for serological tests were obtained at various intervals during the illness. The virus isolation specimens were treated with antibiotics and centrifuged as previously described [43]. The specimens were inoculated into primary dog kidney (PDK) and into the Walter Reed Canine cell (WRCC) line [44]. Negative specimens were subcultured and tested for hemadsorption properties with guinea pig erythrocytes. Procedures for chemical, physical and serologic tests have been described previously [43,44]. Two groups of agents were recovered from this puppy. Toronto A26/61 canine adenoviruses were recovered from the nasal turbinates, lungs, liver, stomach and small intestine of the post-mortem tissues. Virus was not recovered

from the brain, lymph nodes, adrenals, myocardium, pancreas, uterus, spleen, bladder, kidney and colon. Agents were isolated from lungs, small intestine and nasal turbinates in both PDK and WRCC cultures. The other agents were recovered in PDK cell cultures inoculated with specimens obtained 2 days post disease onset. Cytopathic effects (CPE) were seen on the fourth and seventh days in cultures inoculated with rectal and throat specimens, respectively. In both instances the CPE were similar, the infected cells appeared more granular, somewhat "non-specific," and detached from the glass surface. These CPE were readily transmissible. The rectal isolate designated 14-72R was chosen for reference purposes. This isolate was purified by three successive terminal dilutions and seed virus pools were prepared for identification purposes. Cytoplasmic inclusions were evident in stained infected cell cultures. They were eosinophilic on hematoxylin and eosin staining and were green in acridine orange stained cultures. The latter findings indicated the presence of a double stranded viral nucleic acid.

Results of studies on the chemical and physical properties of 14-72R are summarized in Table 6. The isolate was resistant to chloroform and pH 3.0 treatment. Growth of the agent was not inhibited by 5-iodo-deoxyuridine (IUDR) and the agent was readily filterable through 0.45, 0.22, and 0.10 micron millipore filters. However, the virus titer was markedly reduced following passage of virus through the 0.05 micron filter. These findings suggested that the 14-72R agent is naked, contains RNA (probably double stranded) and is less than 0.1 micron in size. On the basis of these findings the isolate could best fit in the reovirus group.

To identify the isolate further, negatively-stained and ultra-thin sections of infected cell cultures were examined by electron microscopy. Dr. A. Strano and Mr. W. Engler of the Armed Forces Institute of Pathology collaborated in these studies. Typical reovirus particles were seen in phosphotungstic acid, negatively-stained preparations. The full and empty virions measured approximately 72 nanometers in diameters. Full and empty core particles of reovirus, 54 nanometers in diameter, were also seen. These core particles are reported to be infectious and thereby explain the infectivity of the 0.05 micron filtrate [45]. Typical reovirus particles and tubules in the cytoplasm were also revealed by examination of the ultra-thin sections of 14-72R infected cell cultures. Full and empty naked particles of approximately 75 nanometers appeared singly and in large aggregates. To identify the reovirus serotype, hemagglutinating antigens were prepared from 14-72R infected PDK cultures maintained in serum-free media. The antigen agglutinated human, but not rhesus or goose erythrocytes.

Table 6. Chemical and physical properties of 14-72R, Canine Reovirus II Isolate.

Treatment	Virus (strain)	1/TCD50 (10)		
		Not Treated (A)	(B) Treated	Change (A-B)
Chloroform	14-72R	7.3	6.7	0.6
	ICH (Cornell)	6.3	6.0	0.3
	Canine herpes (D004)	4.0	< 1.0	> 3.0
pH 3.0	14-72R	6.3	6.8	+0.5
	ICH (Cornell)	5.8	5.5	0.3
	Canine herpes	3.8	< 2.0	> 1.8
IUDR(-3.5M)	14-72R (70 hrs)*	4.1	4.8	+0.7
	SV-5 (Dusty)	4.3	4.9	+0.6
	ICH (Cornell)	3.8	0.0	3.8
<u>Filtration</u>				
**				
0.45 Micron 0.22 Micron 0.10 Micron 0.05 Micron	14-72R	7.0	6.5	0.5
		-	6.0	1.0
		-	5.5	1.5
		-	3.0	4.0

* Increase in virus titer for 70 hours post-infection.

** Millipore membrane filters.

Additional antigens were prepared from the throat (1472T) isolate and from the 14-72R 0.05 micron filtrate. Reference reovirus Types I, II, III antigens and antisera were obtained from the Reference Reagent Branch of the National Institute of Health. The results of the hemagglutination-inhibition (HI) tests are summarized in Table 7. All three 14-72 isolates had the same HI patterns, reacting to highest titer to the Type II antiserum. The reaction to Type II was at least 8-fold higher than to the other reovirus types. The puppy also developed a rise in titer to the reovirus Type II, 14-72R isolate and to reovirus Type III (Table 8). No rise in titer to reovirus Type I was found. Other serological tests did not indicate infection with parainfluenza SV-5.

The findings in the puppy are of interest for several reasons: (a) this is the first recovery from a dog of reovirus Type II; (b) it afforded data on the specificity of antibody response to reovirus II infection; and (c) it presented another case of a multiple viral respiratory infection. The role of canine distemper virus in the observed disease signs is unknown. From the histopathologic examination secondary bacterial invaders were playing a role in the pneumonia.

9. Trachea organ cultures as potential model systems for study of canine viruses.

The tracheal organ culture (TOC) system appears to be a promising tool for the isolation of some of the more fastidious viruses, i.e., coronaviruses and rhinoviruses, and for the study of virus-cell interaction in fully differentiated cells.

Canine TOC of ciliated epithelium have been prepared and maintained in plastic petri dishes for up to 2 weeks with synthetic medium (Leibovitz Medium No. L-15 supplemented with 30% fetal bovine serum and antibiotics). Minor modifications of the methods described by Hoorn and Tyrrell [46] and Kapikian [47] were used. Mucosal cell viability was determined by observation of ciliary activity by the method of Reed [48]; cell viability or injury on the mucosal surface was determined by the use of tetrazolium-trypan blue test of Herbst-Laier [49].

Pilot studies with a number of canine viruses have been completed. The TOC were prepared and infected with canine paramyxovirus SV-5; canine reovirus 14-72R; canine herpesvirus D004; and canine adenovirus Toronto A26/61. Virus proliferation was assessed by inoculation of primary dog kidney tissue cultures with ten-fold dilutions of medium harvests; virus titers were calculated by the Reed and

Table 7. Serologic identification of the 14-72 isolates

Serum tested	Hemagglutination-inhibition antibody titer to antigen					
	Reovirus Type*			14-72 R	14-72R (0.05M filtrate)	14-72 T
	I	II	III			
<u>Reovirus*</u>						
Type I	<u>1280</u>	0	20	20	20	20
Type II	40	<u>640</u>	40	160	160	160
Type III	0	0	<u>320</u>	0	0	0

* Reference reagents obtained from National Institute of Health

Table 8. Antibody response of puppy Z-4 to reoviruses and the 14-72R reference isolate.

Day Post Onset Sera	Hemagglutination-inhibition antibody titer to antigen				
	Reovirus Type			14-72R	
	I	II	III		
2	0*	0	0		<u>0</u>
24	0	20	0		<u>20</u>
31	0	160	80		<u>160</u>
38	0	80	40		<u>160</u>
45	not done	80	20		<u>80</u>
52	0	40	20		<u>40</u>

* less than 1:20

Muench method [50]. Canine TOC's were fixed in either Bouin's fixative or buffered formalin, embedded, sectioned and stained with hematoxylin and eosin.

The results of virus infection indicated that all the above cited viruses can grow or persist in TOC preparations. In these pilot studies each of the viruses were recovered from the TOC media for at least 12 days. Histopathology changes are presently being evaluated.

10. Summary and Conclusions.

A. To elucidate further the pathogenesis of tropical canine pancytopenia (TCP), clinical and hematologic responses of 14 German Shepherd dogs were evaluated following Ehrlichia canis infection, tetracycline therapy, and challenge inoculation. Following an acute phase illness in all dogs, nine developed severe chronic TCP (SCTCP) characterized by severe pancytopenia, hemorrhage, and secondary bacterial infection, while five had non-severe chronic TCP (NCTCP) with only mild pancytopenia. In spite of tetracycline therapy, four SCTCP dogs died while five survived. Hematologic values in surviving SCTCP dogs improved very gradually, thrombocytopenia and leukopenia persisting for at least 120 days post treatment. NCTCP dogs showed only minor hematologic changes following treatment. E. canis could not be recovered from the blood of any treated dog. Six surviving treated dogs, when challenged with the homologous strain of E. canis, became reinfected and two developed severe disease. Results indicate that tetracycline is effective in clearing dogs chronically infected with E. canis and that cleared dogs are susceptible to reinfection. Persistence of pancytopenia following treatment suggests that aplastic anemia may be important in the pathogenesis of SCTCP.

B. Serum antibody and percent gammaglobulin were evaluated in German Shepherd dogs during acute and chronic infection with Ehrlichia canis, following treatment with tetracycline, and after a challenge inoculation of the homologous microorganism. Antibody titers, as determined by the indirect fluorescent antibody (IFA) technique, were detected 7 to 20 days postinfection and continued to increase for more than 2 months to a geometric mean titer (GMT) of 1:120. Mean percent serum gammaglobulin doubled by 26 days postinfection and remained elevated until treatment was initiated. Following administration of tetracycline, GMT values decreased to 1:56 within 100 days, while percent gammaglobulin returned to normal. Increases in IFA titers, as well as hypergammaglobulinemia, were observed following challenge inoculation. Titers in dogs that had developed severe disease following initial infection remained elevated following challenge, whereas titers of dogs not originally having severe disease did not. Results

suggest that increasing IFA titers and hypergammaglobulinemia reflect persistent infection with *E. canis* and may be related to the development of severe disease. Differences in response to challenge may reflect underlying immunologic differences between dogs with and without severe disease.

C. Dogs, cats, and nonhuman primates were successfully infected with an ehrlichial agent recovered from horses in California. Infection was characterized by a mild, transient disease and the presence of morulae in granulocytic cells.

D. *Rickettsia rickettsi* was isolated and propagated in primary cell cultures derived from experimentally infected guinea pigs. Organisms were recognized as early as 3 days after cultures were initiated.

E. Nearly 88% of military dogs procured in 1972-73 were devoid of SV-5 antibody. During the past year no SV-5 infections have been detected at the procurement center. Approximately 76% of the 54 dogs at Ft. Benning were serotest positive for SV-5 antibody. Parainfluenza SV-5 vaccines for use in dogs are undergoing commercial development. The high degree of susceptibility of dogs at the procurement center may warrant the trial of this product to prevent SV-5 respiratory disease in military dogs. Dogs at the Biosensor Unit at Edgewood were found to be also highly susceptible to SV-5 infection. Experimental vaccine trials are recommended in German Shepherd dogs to evaluate the safety and potency of commercial product for this breed.

F. Canine coronavirus isolate 1-71 produced mild gastroenteritis in neonatal dogs. The virus was shed from the rectum for approximately 1 week and the puppies developed antibodies to 1-71 and TGE. In contrast, neonatal and young pigs were resistant to 1-71 virus. Following TGE challenge pigs developed higher titers to TGE than 1-71 virus. The 1-71 isolate thus appears to be distinct from TGE and may be a causative agent for diarrheal disease in dogs.

G. Reovirus Type II was recovered from throat and rectal specimens obtained from a puppy in the acute stage of respiratory disease. This virus had not been previously reported in dogs. The puppy developed an antibody rise to the isolate and to reovirus Types II and III. A Toronto A26/61 canine adenovirus was recovered from the post mortem tissues of this puppy.

H. Canine trachea organ cultures (TOC) have been prepared and were maintained for 2 weeks. Initial growth studies of other canine viruses were also carried out. The TOC system appears to be a valuable system for studying the pathogenesis of canine viruses and further studies with this system will be carried out.

Project 3A062110A830 BIOSENSOR SYSTEMS

Task 00 Biosensor Systems

Work Unit 056 Diseases of military animals

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PROJECT 3A062110A833
BIOMEDICAL FACTORS IN DRUG ABUSE

Task 00
Biomedical Factors in Drug Abuse

1234

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1 AGENCY ACCESSION ^a	2 DATE OF SUMMARY ^a	REPORT CONTROL SYMBOL DD DR&E/AR)636	
3 DATE PREV SUMMARY	4 KIND OF SUMMARY	5 SUMMARY SCTY ^a	6 WORK SECURITY ^a	7 REGRAOING ^a	8A DISB'N INST'N	8B SPECIFIC DATA CONTRACTOR ACCESS ^a	9 LEVEL OF SUM
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A. PRIMARY	621101A	3A062110A833		00		101	
B. CONTRIBUTING							
C. OTHER	CDOG 114(f)						
11 TITLE (Precede with Security Classification Code) ^a							
(U) Drug Abuse Methodology							
12 SCIENTIFIC AND TECHNOLOGICAL AREAS ^a							
002300 Biochemistry							
13 START DATE		14 ESTIMATED COMPLETION DATE		15 FUNDING AGENCY		16 PERFORMANCE METHOD	
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17 CONTRACT GRANT				18 RESOURCES ESTIMATE		19 PROFESSIONAL MAN YRS	
A. DATES/EFFECTIVE NA				B. PREC'D JN'S		C. FUNDS (in thousands)	
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C. TYPE				73		12	
D. KIND OF AWARD				74		280	
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20 RESPONSIBLE DOD ORGANIZATION				21 PERFORMING ORGANIZATION			
NAME * Walter Reed Army Institute of Research				NAME * Walter Reed Army Institute of Research			
ADDRESS * Washington, D. C. 20012				ADDRESS * Washington, D. C. 20012			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)			
NAME. Buescher, COL E. L.				NAME * Beach, LTC D. J.			
TELEPHONE 202-576-3551				TELEPHONE 202-576-2211			
				SOCIAL SECURITY ACCOUNT NUMBER [REDACTED]			
22 GENERAL USE				ASSOCIATE INVESTIGATORS			
Foreign Intelligence Not Considered				NAME Demaree, LTC G. E., Kazyak, L., MS			
				NAME Bass, B.C., MS Tripp, MAJ C. H. DA			
23 RE * W.P.D.S (Precede SSAN with Security Classification Code) (U) Mass spectrometry							
(U) Drugs of abuse; (U) Toxicology; (U) ESR; (U) Chromatography; (U) Immunoassay							
24 TECHNICAL OBJECTIVE, 25 APPROACH, 26 PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code)							
23. (U) To develop and assess analytical techniques for the detection, identification and quantitation of drugs of abuse in biological fluids for use in the military population.							
24. (U) Efforts will be concentrated on the development and optimization of simple, rapid and accurate systems of drug detection for operational laboratory use. Some of the techniques utilized will be thin layer chromatography (TLC), gas liquid chromatography (GLC), high pressure liquid chromatography (HPLC), free radical assay technique (FRAT), mass spectrometry (MS), radioimmunoassay (RIA), hemagglutination inhibition (HI), and enzyme multiplied immunoassay technique (EMIT). Use of appropriate quality control measures will be stressed.							
25. (U) 72 07 - 73 06 Drug screening methodology development and quality control assessment of commercial contract laboratories continued. An organic chemistry laboratory to synthesize drugs and metabolites of interest, was established. Use of HPLC was initiated. Use of RIA, EMIT, and HI was investigated. Drug screening was performed in collaboration with other WRAIR divisions to examine various drug abuse parameters. For technical report, see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 72 - 30 June 73.							

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Project 3A062110A833 BIOMEDICAL FACTORS IN DRUG ABUSE

Task 00 Biomedical Factors in Drug Abuse

Work Unit 101 Drug abuse methodology

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The technical objectives of this work unit are to develop and evaluate analytical methods for the detection, identification and quantitation of drugs of abuse in biological fluids and to exploit these techniques for application to mass screening and rehabilitation in military populations.

Efforts were concentrated in these areas.

1. Free Radical Assay Technique (FRAT).
2. Thin Layer Chromatography (TLC).
3. Testing of Commercial Kits and Systems.
4. Screening for drug abuse in selected military populations.
5. Quality Control Support for the Army Urine Screening contracts.

1. FRAT

FRAT analysis utilizes an electron spin resonance (ESR) spectrometer which has been modified for the testing of drugs in protein-free biological fluids. Various factors can influence morphine detection by this procedure and have been investigated. The investigation of the use of the FRAT to detect drugs other than morphine was continued.

Reducing agents normally present in urine can decrease the signal response observed by modifying the spin label. Therefore, sodium dichromate is added to negate the effect of the reducing agents. Studies

conducted to determine the optimum amount of dichromate to be added indicated optimum concentration of dichromate for most assays to be 0.2 M. Concentrations of dichromate above 0.4 M were shown to decrease the signal and hence invalidate the quantitation of morphine.

a. Aqueous solutions of 90 commonly used drugs were analyzed for possible reactivity in the FRAT assay for morphine. Of the drugs tested, 18 gave positive reactions. They were quinine, methadone, codeine, meperidine, diphenylhydantoin, cocaine, propoxyphene, meprobamate, oxymetazoline, chlorpromazine, chloroquine, heroin, penicillin G, procaine penicillin, mephentermine, hydralin, ephedrine, haloperidol, and benzathine. Efforts are being made to obtain urine from individuals taking commonly used drugs to determine if the parent compound and/or metabolites give morphine equivalent values.

b. The use of newly developed reagents for the FRAT to determine other drugs of abuse was investigated.

1) Amphetamines. The assay for amphetamines in urine was unsatisfactory because normal urinary amines react with the antibody. This limits the usefulness of the test for screening.

2) Amphetamine-cocaine metabolite combined assay was technically difficult to perform. The test is subject to large numbers of false positive responses.

3) Methadone reagents react with the parent compound but not its metabolites with a detection limit of 0.50 $\mu\text{g/ml}$. Propoxyphene, chloroquine, primaquine, procaine penicillin and chlorpromazine react with the antibody. Methadone was detected in human urine for up to 3 days after a 50 mg dose.

4) The methadone-barbiturate combined assay was technically difficult to perform and the stability of the two spin-labeled drugs employed were not the same. No reliable quantitation of drug concentration could be made since the ESR signals of the two drugs are indistinguishable and the antibodies have different binding kinetics.

5) Work was initiated to automate drug analysis using ESR spectrometry. Preliminary design of the instrumentation and evaluation of reagents are in progress. Application of the Varian computer to control and collect data produced by the ESR spectrometer is being evaluated. Preliminary indications are that the Varian (VDM 620) computer obtained for use in this project lacks flexibility for optimum utilization. Additional core memory will be required for realization of the full potential of this system.

6) Characterization of FRAT antibody to morphine.

a) Two titrations of the SYVA antibody to morphine were performed using spin-labeled morphine (SLM) and ESR spectrometry. When SLM was added to 500 μ l (2.2 mg protein) of FRAT antibody an equivalence point of 1.7×10^{-8} moles SLM/mg protein was observed. These data indicated an antibody-SLM dissociation constant of 1.5×10^{-10} . The reverse titration (antibody added to 2.6×10^{-3} μ moles SLM) indicated an equivalence point of 1.8×10^{-9} moles SLM/mg protein and a dissociation constant of 2.1×10^{-10} .

b) The competitive reaction between SLM and free morphine for a binding site on morphine-specific antibody binding constant, K , then batch-to-batch comparisons should be possible if either the association constant, K_A or K_D respectively, for each lot of antibody is known.

The association of the hydrophobic fluorescent probe, 2-p-toluidinyl-6-naphthalene sulfonate, (TNS), with the SYVA antibody preparation was studied. Protein concentration of the antibody solution was found to be 4.4 mg/ml as determined by the Lowry method. Agar gel electrophoresis showed the gamma globulin concentration to be 3.8 mg/ml or 87% of the total protein. Immunodiffusion using human serum antibody identified the gamma fraction as IgG.

TNS binding data gave the total number of binding sites, n , and the statistical thermodynamic dissociation constant, K_D 25:

$$\begin{aligned}n &= 1.66 \times 10^{-5} \text{ moles/ml total protein} \\&= 9.99 \times 10^{18} \text{ sites/mg total protein} \\K_D &= 1.23 \times 10^{-7} \text{ moles}\end{aligned}$$

2. TLC

TLC is a versatile technique for the rapid detection of a wide range of drugs in human urine. The TLC method employed for urine screening was a modification of one proposed by Davidow, Petri and Quame using an organic solvent extraction (chloroform-isopropanol) of urine buffered to pH 9.5. Amphetamines, barbiturates, morphine, codeine, methadone and other drugs are detected by using several visualizing sprays.

a. An evaluation of solvent systems revealed that with urine buffered with ammonium chloride/ammonium hydroxide at pH 9.0-9.5, chloroform alone extracted barbiturates and amphetamine and extracted less interfering substances. This modification reduces the sensitivity for detection of morphine and codeine below levels of 1.0 μ g/ml. Opiates are screened by FRAT, thereby eliminating the dependence on TLC for opiate screening. Investigations of various identifying sprays and developing solvents are proceeding.

b. Selected prescription and over-the-counter drugs are being studied to determine which are excreted into urine and may interfere with this TLC system. Urines from humans currently taking antihistamine, analgesic, sedative, stimulant, tranquilizer, and others are being examined by TLC. The R_f 's and colors produced by the identifying sprays are recorded and tabulated for reference in drug screening studies. Comparative studies using urine from animals have been initiated.

c. The investigation of TLC procedures for the detection of methaqualone was initiated. The methods developed to date lack sensitivity or reproducibility for use in drug screening laboratories.

3. Testing of Commercial Kits and Systems.

Upon direction of TSG and CG, USAMRDC, commercial kits were evaluated for application to mass urine screening and for use in rehabilitation laboratory support. Kits were evaluated for cost, reliability, sensitivity, specificity, logistical requirements, and technical applicability. Kits and systems presently under investigation include Narco Med Pack, Esco Kit, Toxi X, EMIT, FRAT, HI, and RIA. HI and RIA are presently restricted to morphine screening; FRAT and EMIT are claimed to be effective for amphetamines, barbiturates, methadone, and cocaine metabolites. RIA for morphine is supplied for either H-3 or I-125 label. These evaluations are still in progress. At this time no kits or systems appear to offer advantages that would warrant replacement of the FRAT system.

4. Screening for Drugs of Abuse in Selected Military Populations.

a. A time-motion study was conducted using 807 urine specimens collected from Fort Lee, Va., and 41 quality control samples that were inserted at random among the urine samples. FRAT, RIA, HI, TLC, and GLC were compared.

- 1) HI is the fastest for opiate screening.
- 2) HI requires no special equipment and is simple to perform.
- 3) The RIA results were similar to those of HI. RIA requires a large time expenditure compared to FRAT or HI.
- 4) All three screening tests for opiates (FRAT, HI, RIA) respond to codeine and they can be expected to respond to all opiate-like substances.
- 5) Confirmation of drugs is based on the use of GLC - a slow and cumbersome technique.
- 6) TLC is cumbersome and it is at the limit of its capability as a screening technology.

7) Both RIA and HI can be used in segments of the urine screening program. RIA could be used effectively where counting equipment is already available, particularly in large hospitals having a rehabilitation and treatment program. HI, because of its simplicity and low equipment requirements can be used in small medical facilities and for the survey of isolated units.

b. Two experimental serological assays for opiates, RIA and HI were compared with two established methods, FRAT and TLC on the basis of cost, efficiency, and efficacy using the samples from an operational screen verified by GLC for comparison.

1) Samples for this study were withdrawn from the sample bank maintained by the U.S. Air Force Drug Screening Laboratory, Epidemiology Branch, School of Aerospace Medicine, Brooks Air Force Base, Texas. All samples were screened by TLC and positive samples were shipped to WRAIR for analysis.

2) All three serological tests exceeded 99% performance in the detection of samples containing opiates. RIA had a lower rate of cross-reactivity with other drugs than HI or FRAT. It was concluded that any of the three immunoassay methods is satisfactory for use in screening for opiates in urine and that they all offer operational advantages over TLC.

c. Urine specimens collected in RVN under the direction of MAJ M. G. Robinson, MC, were analyzed for opiates by FRAT approximately 6 months after collection. They were re-analyzed at 12 months following collection to determine opiate stability. The urine was stored frozen in plastic bottles but had been thawed and refrozen numerous times. Of the original 354 samples, 342 were available for comparative analyses at 6 and 12 months after collection. A comparison of the FRAT results 6 and 12 months post-collection revealed that 2.9% of the original positive samples were analyzed as negative after 12 months. Nine per cent of the positive specimens retained the original response and 91% of the positive responses gave relative decreases in the morphine response after storage. The reasons for this decrease are being investigated.

d. In a collaborative study with the Division of Neuropsychiatry, WRAIR, urines collected at Fort George G. Meade, Md., and an appropriate number of controls were analyzed by FRAT and TLC. Of the 3956 urines collected, 3567 were negative by both methods, 268 were positive, and 120 were designated as + by FRAT. Some urines gave positive responses or + responses for more than one drug. The total number of urines found positive for each drug and the percentage of the total urines is shown in the following table.

Drug or Test	No. Positive ¹	% Positive	No. <u>+</u> ²	% <u>+</u>
FRAT	92	2.3	134	3.4
Amphetamine	12	0.3	0	-
Barbiturate	42	1.0	0	-
Morphine (TLC)	18	0.4	0	-
Codeine	32	0.8	0	-
Methadone	10	0.2	0	-
Others	72	1.8	0	-

¹ Minimum detection: 100 ng/ml

² Minimum detection: 50 ng/ml

1) HI was performed on 3832 of the samples. There were 73 positive urines and 2 + responses. All morphine and codeine quality control specimens were positive by the HI test.

2) The RIA (Abuscreen) test was performed on 1127 selected Fort Meade samples, 27 gave positive responses and 14 gave + responses. Morphine and codeine quality control urine samples were identified 41 out of 42 times. The RIA test gave one positive response with a methadone quality control specimen.

3) The FRAT, HI and RIA were compared in 1125 Fort Meade specimens of which 103 were positive by one or more of these tests. The results found for the positive urines are shown in the following table.

Test	Response			% Positive
	-	<u>+</u>	+	
FRAT	21	50	32	2.7
HI	75	2	26	2.2
RIA	61	14	28	2.4

Since the sensitivity and specificity of the methods are different, direct comparison of reactivity is difficult. However, the HI test appears to be the most specific, and the FRAT the least specific of these methods when the + responses are considered at these levels.

e. Urine from patients who were given heroin or morphine were obtained from the Addiction Research Center, Lexington, Ky. The samples were collected between March and August 1971, and were analyzed by FRAT in December 1971. The same samples were re-analyzed in March 1973. In general, the values of morphine equivalents reported in 1973 are lower. In 1971, fluctuating low levels of morphine

were observed by FRAT in urine collected 48 hours and longer after administration of the drug. In 1973, only three samples gave any positive responses after 48 hours. Selected samples were analyzed by GLC or GC-MS in March 1973, for the presence of morphine. These values gave excellent agreement with the FRAT values of March 1973, and in general, failed to detect urinary morphine after a lapsed time of greater than 48 hours after a single 5 mg dose of heroin.

5. Quality Control Support for the Army Urine Screening Contracts.

The Division of Biochemistry was given the responsibility for setting up a quality control assurance laboratory in support of the U.S. Army Medical Department's contracted drug screening effort. This responsibility involved the preparation, analysis, shipment and post-analysis of urine specimens used by the contracting office to determine the level of compliance of the various commercial laboratories under contract for drug screening efforts. This laboratory has been operating for the past year in response to the needs of the contract program. Approximately 4,000 specimens were prepared and distributed.

Project 3A062110A833 BIOMEDICAL FACTORS IN DRUG ABUSE

Task 00 Biomedical Factors in Drug Abuse

Work Unit 101 Drug abuse methodology

Literature Cited.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION ^a	2. DATE OF SUMMARY ^a	REPORT CONTROL SYMBOL DD-DR&E(AR)6.36	
3. DATE PREV. SUMRY	4. KIND OF SUMMARY	5. SUMMARY SCTY ^a	6. WORK SECURITY ^a	7. REGRADING ^a	8. DISSEM INSTRN ^a	9. SPECIFIC DATA - CONTRACTOR ACCESS ^a	10. LEVEL OF SUM ^a
	A. New	U	U	NA	NL	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	A. WORK UNIT
11. NO. CODES ^a	PROGRAM ELEMENT	PROJECT NUMBER		TASK AREA NUMBER		WORK UNIT NUMBER	
A. PRIMARY	62110A	3A062.10A833		00		102	
B. CONTRIBUTING							
C. OTHER PERSONS WORK	CDOG 114(F)						
11. TITLE (Precede with Security Classification Code) ^a							
(U) Military Performance and Drug Abuse							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS ^a							
013400 Psychology 012600 Pharmacology 012900 Physiology 003500 Clinical Medicine							
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NAME: Walter Reed Army Institute of Research				NAME: Walter Reed Army Institute of Research			
ADDRESS: Washington, D. C. 20012				ADDRESS: Washington, D. C. 20012			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)			
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21. GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER			
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				NAME: Sogatz, CPT F. J.			
				NAME: Hegge, F. W., Ph.D.			
				DA			
22. KEY WORDS (Precede EACH with Security Classification Code)							
(U) Delta-9-THC; (U) Heroin; (U) Drug Abuse; (U) Performance; (U) Withdrawal; (U) Addiction; (U) Tolerance; (U) Drug Secretion							
23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
23. (U) The mission of this unit is to identify environmental, behavioral, and physiological factors associated with the abuse of drugs which lead to ineffective military performance, prolonged addictive disease, or dangerous toxic effects.							
24. (U) Research methods of psychology, psychophysiology, physiology, biochemistry, neuropharmacology, and clinical evaluation are used to identify and correct the untoward effects of drug abuse upon the performance and health of military personnel.							
25. (U) 72 07 - 73 06 In usual dosage ranges, chronically administered Delta-9-THC, the principal agent in hashish and marihuana, produces transient deficits in performance under conditions which produce disadvantageous consequences for the organism; drug induced changes are sustained when they cause no immediate disadvantage. The behavior decrement due to marihuana will vary in response to the immediate consequences of environmental reinforcement. The time course of brain self-stimulation and neurochemical changes accompanying acute euphoriant effect of heroin has been partially analyzed. Although very high doses of pure heroin were consumed by addicts, the resultant acute withdrawal syndrome was mild. Potentially important, prolonged, subtle physiological effects are present. The probable persistence of heroin and/or its metabolites over a 14-day period following withdrawal has been established. These findings modify concepts of the nature of early opiate addiction in humans. For technical report, see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 72-30 Jun 73.							

^a Available to contractors upon originator's approval

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Project 3A062110A833 BIOMEDICAL FACTORS IN DRUG ABUSE

Task 00 Biomedical Factors in Drug Abuse

Work Unit 102 Military performance and drug abuse

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Description.

Under this work unit, the work conducted concerned the impact of drugs of abuse upon human performance and health. This research has been carried out in clinical and laboratory situations on human patients, primate laboratory models and in experimental systems utilizing other species. During the last year, work concentrated on the effects of heroin and delta-9-tetrahydrocannabinol, the principal ingredient of marijuana and hashish. Studies have concerned themselves with critical environmental and physiological changes which are likely to lead to disruptions of performance or sickness. (See also Work Units 102, 077, 122 and 025).

I. HEROIN DEPENDENCE AND WITHDRAWAL IN THE MILITARY HEROIN USER IN THE U.S. ARMY, VIETNAM (USARV).

Following the data collection phase of studies of heroin use, dependence and abstinence syndrome in Vietnam (April through June of 1972), data analysis has been undertaken, allowing presentation of preliminary findings. Data were collected on a 24-hour basis in a controlled environment at the U.S. Army Hospital, Long Binh, Vietnam, on 31 heroin users and 5 non-heroin users (control group). By design, the object of this study was to characterize the pharmacophysiologic response of recently addicted (1 year or less) individuals to heroin and withdrawal. Most data are derived from a sample of 10 individuals who met the study criterion and 5 controls. Intense observations included 24-hour electrophysiologic and clinical monitoring and were made for approximately 7 days; other less intense sampling procedures (e.g., regular blood and urine sampling) continued for a total of 14 days.

A. Biochemical and Behavioral Findings During Acute Heroin Abstinence in Vietnam.

Differences between heroin users in Vietnam and those classically described in civilian reports were documented. Such findings included the following distinctive parameters for the military heroin abuser in USARV: youth, relatively brief exposure to opiates, good general health, use of nasopulmonary routes of heroin administration rather than intravenous or other hypodermic self-injection, remarkably mild withdrawal symptomatology (especially when evaluated relative to the tolerance of many of these patients to very large quantities of heroin), and the ability to evaluate withdrawal without the need for therapeutic pharmacological intervention. Gross clinical scaling of withdrawal (abstinence syndrome) severity confirmed the previously unsubstantiated impression of its benignity and brevity. Free Radical Assay Technique (FRAT) measurement of "morphine equivalents" in 24-hour urine collections from withdrawing patients established an index of drug excretion never previously available in a comparable study population. At least by this general immunochemical technique, early morphine and metabolite excretion fell from initial, extremely high levels in an exponential pattern, reaching a variable "plateau" after 5-6 days. Of particular importance is the observation that morphine or morphine metabolite excretion continued to be found as late as 14 days following the last dose of heroin. Pupillometry performed 3 times daily during the course of abstinence documented the pattern of early miosis and late mydriasis typical of classical opiate withdrawal; however, along with morphine excretion, pupil size remained abnormal well beyond other signs and symptoms of abstinence.

B. Acute Heroin Withdrawal in Vietnam; Biochemical and Clinical Findings: A Preliminary Report.

1. The extreme purity of heroin in Vietnam (92-98%) has been stressed along with the freedom of the study group from concomitant use of other illicit drugs (except marijuana). Modification of the FRAT for determination of "morphine equivalents" at levels below 100 nanograms per milliliter was accomplished by the Division of Biochemistry, WRAIR; and the utility of the techniques has been documented. The estimate of heroin dose by clinical history can be substantiated by such assays of total immunologically recognizable morphine and metabolite excretion. Preliminary morphine excretion data suggest that the insufflation of heroin intranasally (snorting, sniffing) results in absorption of heroin

of approximately the same magnitude as that associated with intravenous injection. Smoking of heroin results in somewhat lower initial excretion of morphine equivalents in the patient's urine (a finding in accord with earlier studies of the heroin-using civilian population in Hong Kong by Quock and Way, 1968). Of interest, no consistent overall difference in morphine equivalent excretion can be attributed to route. The definition of possible qualitative differences (e.g., metabolite patterns) awaits development of appropriate analytical techniques aside from the several immunochemical approaches applied to date.

2. Although the subjects studied reported high heroin intake (which was documented by immunochemical urinalysis), the abstinence symptomatology in Vietnam had a total duration of less than half the period reported in civilian "cold turkey" withdrawal. Further, the specific signs and symptoms observed in the withdrawing Vietnam soldier were considerably less severe than those commonly reported in civilian heroin addicts. These clinical findings support an "uncoupling" of tolerance and physiological dependence. If this is supported by further analysis, the previous assumptions about the interdependence of tolerance and addiction must be revised.

C. Pupillary Responsivity During Acute Heroin Withdrawal In Viet Nam.

Further analysis of pupillary measurement data from Vietnam has provided clearer indices of this physiological response to opiates and their withdrawal. The miosis of acute heroin intoxication did not increase with light-stimulation or in response to accommodation. In the 12-18 hours following the self-administration of the patients' last doses of heroin, mydriasis gradually developed (all measurements considered in relation to concurrently evaluated drug-free controls). Pupillary dilatation persisted throughout five days of measurement, therefore extending beyond the 2-3 days of symptomatic clinical abstinence syndrome. Although the dilated pupils of withdrawing patients did respond to light with some constriction, divergence from similarly simulated controls also persisted. A Linquist Type VI analysis of variance indicated a consistent circadian variation in both patients and controls, maximum pupil diameter occurring in the late evening and minimum size in the early morning. This finding has not previously been reported in the English language scientific literature.

D. Hemagglutination Inhibition Assay For Morphine.

In collaboration with Dr. Frank L. Adler (Chief, Department of Immunology, Public Health Research Institute of the City of New York) more than 5,000 samples of urine and serum from Vietnam have been assayed for morphine equivalents by the hemagglutination inhibition technique. This technique is complementary to FRAT and has allowed independent assessment of morphine equivalent kinetics in Vietnam studies. Hemagglutination inhibition can be used to measure morphine equivalents in serum or plasma. This allows preliminary formulation of a schema for morphine metabolism in this patient population, which is critical for design of more specific chemical analyses of these specimens. Preliminary results of such assays in selected RVN patients indicate the continued intermittent presence of circulating morphine equivalents for more than two weeks following the last dose of heroin. All hemagglutination inhibition assays have been done under "double blind" conditions with a minimum of three dependent replications of each sample. Continued collaboration is planned in the analysis of existing data.

E. Clinical Chemistries In Vietnam Heroin Users And Control Subjects.

1. Blood Chemistries.

Tests have been performed at Hycel Corporation in collaboration with LTC Douglas Beach, MC, Director, Division of Biochemistry, on the first series of clinical chemistries from the Vietnam investigations. Tests completed include: creatinine, calcium, phosphorus, LDH, CPK, SGPT, SGOT, alkaline phosphatase, total bilirubin, sodium, potassium, cholesterol, uric acid, total protein, globulin, BUN and glucose. Although findings are still preliminary, some differences between the heroin users and controls have emerged. Heroin users have significantly lower serum values for: creatinine, cholesterol, uric acid and BUN. This may well be secondary to impaired nutrition and decreased lean muscle mass among the drug users. Heroin users have higher phosphorus and CPK than controls. There is also an apparent tendency toward lowered serum sodium and slightly increased blood glucose among the heroin users (hyperglycemia has been described as a physiological effect of the narcotic drugs). Further evaluations of present data and additional clinical laboratory studies are underway.

2. Urinary Chemistries.

Sequential urine specimens from 31 heroin users and 5 controls have been analyzed in collaboration with Dr. James Low of the Metabolism Section, Walter Reed General Hospital. Over 400 samples have been assayed for sodium, potassium, creatinine and osmolarity (in duplicate). Data analysis is underway to correlate urinary clearances of electrolytes and creatinine with morphine excretion and with the course of abstinence syndrome.

3. Hematological Findings.

On the study population and controls, data are available for white blood cell count, differential percentages of leucocyte types, blood cell morphology, hematocrit, prothrombin times and sedimentation rate. Most cases provide values three times daily during withdrawal. Initial evaluation of results indicates a persisting leucocytosis during withdrawal; however, the normal circadian pattern of white cell count seems to be preserved (maximum during the evening and minimum during the morning hours).

F. The Analysis Of Continuous Heart Rate, Respiration Rate, Electroencephalogram And Gross Eye Movements.

1. Electrophysiologic data were collected by telemetry on a 24-hour basis for 5 to 7 days. Techniques have been developed for decomposing two channels of recorded electrophysiological data into their constituent signals. Signals from a pair of transdiaphragmatic electrodes were filtered to produce electrocardiograms (ECG) and rheopneumograms (RPG). Signals from scalp electrodes were filtered to produce electroencephalograms (EEG) and electro-oculograms (EOG). Magnetic data tapes were played at faster-than-recording speed to provide time compression, thereby shortening analysis time.

2. The preliminary analysis done to date has concentrated on the gross characteristics of these signals, i.e., on the statistical descriptors specifying rate of occurrence and signal variability. These data are from long time-series that extend from 5 to 7 days in duration. The manipulation of the long data files required the development of special analytic and graphical presentation techniques. Sufficient material has been examined to date to permit a preliminary characterization of the differences between addicts undergoing withdrawal and yoked normal controls.

3. Gross eye movements are considered to be related to the general activity of a subject. Normal subjects exhibit a clear diurnal pattern of activity with 3 or 4 subsidiary activity periods between 0600 and 2400. Addicts differ markedly from controls in that the diurnal activity pattern disappears and is replaced by higher levels of activity that continue throughout the day. The normal diurnal patterns are not reestablished by day 6 of withdrawal, although overall activity levels return virtually to normal levels. These data provide a precise means of analyzing the temporal structure of activity during withdrawal from heroin. These analyses are proceeding.

4. Little has been done with the respiration data to date other than to digitize, filter and store signals for subsequent analysis. An adaptive peak detector program has been written and debugged for that analysis. The program samples noise levels, and on the basis of the statistical properties of the noise, sets variable threshold values which reduce the probability that spurious signals will be classified as breaths. This step was necessary because the recording technique is extremely sensitive to changes in electrode pressure such as occur during the monitoring of free-ranging subjects.

5. The analysis techniques for the ECG data are most highly developed and have been employed most extensively. Each individual cardiac interbeat interval is collected over 10-minute periods. Within each 10-minute period the intervals are partitioned directly into frequency distributions, or they are converted to instantaneous heart rate and then partitioned. Descriptive statistics are calculated on each of the 10-minute distributions and their values become time series subjected to further analysis. Normal subjects show clear and expected circadian and ultradian variation in interbeat interval. The addicts, on the other hand, show a marked suppression in both circadian and ultradian activity early in withdrawal. Ultradian activity returns before circadian activity. These findings have important implications for our understanding of central neural control mechanisms and heroin detoxification.

G. Miscellaneous Continuing Investigations.

In collaboration with the Division of Biochemistry, arrangements for quantitative/qualitative analyses of our biological specimens for morphine and other metabolites have been planned. In collaboration with the Department of Neuroendocrinology, serial urine samples and plasma specimens are being assayed for an endocrine battery to define the effects of narcotic

usage and withdrawal in this population and setting on the hypothalamic-pituitary-adrenal axis, endocrine pancreas, thyroid and gonadotrophic-gonadal hormone systems. (Work Unit 077, Influence of stress on hormone response, performance and emotional breakdown in the military).

II. CLINICAL STUDIES PERTAINING TO HEROIN USE IN USARV.

A. Medical histories on approximately 2500 USARV soldiers, non-heroin users and a similar number of heroin users, were obtained. Frequency analyses of these data are being prepared. In addition, data pertinent to admissions to USARV Drug Treatment Centers are being studied and correlated with aspects of demographic variables including items pertinent to heroin history.

B. A frequency analysis of signs and symptoms associated with heroin abstinence is being prepared on approximately 500 soldiers who were admitted to USARV Drug Treatment Centers. One hundred forty of this group in addition underwent careful, daily neurological examinations during withdrawal.

C. A review of medical complications associated with heroin use and a review of autopsy data pertinent to drug and alcohol abuse in USARV in 1970, 1971 and the first half of 1972 have been completed and are now being further evaluated.

III. LABORATORY STUDIES ON HEROIN USING SOLDIERS IN USARV.

A. Hematology Studies.

Peripheral blood smears obtained in USARV for the study of white cell morphology in 230 heroin users and a similar number of non-users are being carried out in cooperation with the Department of Hematology, WRAIR.

B. Hepatitis Associated Antigen Studies.

Sera from 300 heroin users and 200 non-heroin users from USARV have been studied for presence of HBAG and HBAB by RIA in the Department of Hematology. Results are being correlated with pertinent demographic and drug use data.

C. Immunological Studies.

Immunoelectrophoretic patterns are being carried out on sera from 100 USARV heroin users. This is being done because of previous reports

of IGM elevations in CONUS heroin users. It is also planned to study these same sera for presence of morphine antibody. These studies are being done in cooperation with the Division of Biochemistry, WRAIR and the Department of Pathology, WRGH.

D. Morphine Excretion Studies.

To document metabolic and excretion patterns, sera and urines were collected at frequent, regular intervals from 75 individuals who smoked, insufflated, and injected heroin. These specimens will be analyzed for heroin and its metabolites.

E. Pulmonary Function Studies In Vietnam Heroin Users And Control Subjects.

Pulmonary function testing was performed on 145 identified heroin users and drug-free staff members in the Long Binh Drug Treatment Center and Crossroads Rehabilitation Center. One-second forced expiratory volume, 3-second forced expiratory volume, peak expiratory flow, and total vital capacity were measured. Height, weight, age, drug history (including marijuana use), medical history, and demographic information were obtained for all subjects. Blood was obtained from each participant at the time of pulmonary function testing for morphine/metabolite level determination, clinical chemistries, hepatitis antigen determination, hepatitis antibody determination and immunoglobulin assay. Preliminary pulmonary function test results indicate impairment of expiratory flow correlated with recent use of heroin. Further data analysis will include assessment of pulmonary function in relation to serum morphine level, smoking history, heroin route, height, weight, length of tour in Vietnam and tendency toward recovery of "normal" pulmonary function values on follow-up testing.

IV. PERFORMANCE EFFECTS OF DELTA-9-TETRAHYDROCANNABINOL (THC).

A. Effects Of THC On Timing Behavior: Acute And Chronic Administration.

Rhesus monkeys were trained to lever press for food reinforcement on a differential reinforcement of low rates (DRL) schedule, then given six different doses of THC ranging from .07 to 2.86 mg/kg at weekly intervals. Relative to controls, all six doses produced increases in both the number of unreinforced responses, that is, errors and the time re-

quired to obtain 60 reinforcements, as well as decreases in the median interresponse time (IRT). In addition, marked pausing occurred after higher doses. These performance changes were occasionally evident 24 hours later, but more often were less prominent in the second half of the session and were followed the next day by sessions with exceptionally long interresponse time, and exceptionally few errors. Starting sessions four hours after THC ingestion, rather than three hours afterwards, altered neither number nor distribution of errors. Apparently, it is the opportunity to practice performance under the influence of THC since last drug dose that is responsible for improvement seen during drug sessions. This suggestion is supported by the ease with which drug-induced performance changes, within session recovery, and subsequent improved performance can be mimicked by merely increasing the minimum reinforced interresponse time from 60 to 72 seconds. Upon completion of the procedure described above, the same animals were given daily doses of 1 mg of THC. At least partial tolerance to the effects described above was seen in all animals. In addition, on discontinuation of daily THC, after complete tolerance or thirty days, a marked rebound was observed. Tolerant monkeys showed interresponse times significantly longer during the initial few post-drug sessions than in the pre-drug baseline period. Continued post-drug testing resulted in a return to the IRTs typical of the baseline period. These studies on the effects of THC on spaced responding demonstrate that behavioral effects can be seen in the monkey at doses typical of human usage, that DRL schedules provide especially sensitive baselines for investigating this drug, and that at least under some conditions rapid behavioral compensation for drug induced decrements in performance is both possible and likely.

B. Effects Of Chronic Administration Of THC On Work Rate Of Accuracy Of Performance And Timing Behavior.

A series of studies were completed during which three chimpanzees were required to work on a task that required precise timing behavior, as well as sustained performance for nearly 16 hours each day in order to obtain their daily ration of food. THC was administered orally on each of the 35 successive days. Doses of THC comparable to those reported effective in humans resulted in over-estimation of time and a reduction of work output. Gradual recovery from this deficit occurred during the first 14 successive days of THC administration; throughout the remainder of the administration period, behavior was essentially normal. When the drug administration ceased, a marked decrement in performance was observed which lasted approximately 14 days. This decrement was gradually eliminated with successive post-drug sessions. Thus, with chronic

administration of THC, a deficit in performance is observed that is gradually eliminated as the organism continues to perform in a manner consistent with the schedule of reinforcement. When the drug is no longer administered, another deficit in performance occurs which is also eliminated with continued exposure to the schedule of reinforcement. These observations suggest that tolerance to THC occurs when the effect of the drug leads to disruption or decrement in performance, but does not occur in those special situations in which performance enhancement is seen following drug administration. Both phenomena are consistent with the notion that behavioral tolerance to THC may reflect the continued operation of the schedule of reinforcement used to maintain performance.

C. Tolerance To The Effects Of THC On Free-Operant Avoidance.

Eight animals were trained to lever press to avoid shock in a free-operant situation with an response-shock interval of 20 seconds and shock-shock interval of 2.5 seconds. Asymptotic performance ranged from near-perfect avoidance to mere post-shock responding. THC given orally 4 hours before testing, initially produced an increase in the number of shocks received. With continued daily exposure to the drug, this increase waned, disappearing within seven days for all subjects. In addition, with the exception of two extreme subjects, shock rates continued to decline until substantially lower than the pre-drug baseline. This reduction was not accompanied by an increase in response rate, but was due primarily to a change in response spacing. More importantly, this improvement did not disappear until drug administration was discontinued 14 days after the first sign of improved performance. At this point, performance returned to pre-drug levels.

D. Circadian Variation In The Effects Of THC On The Temporal Control Of Performance.

Monkeys are being trained on a multiple schedule with both fixed-interval and DRL components. Four sessions are run daily, two in the lighted portion of the day and two in the dark. THC in doses from .5 to 2 mg/kg are being given two hours before each session in an attempt to determine the relationship between performance decrements attendant to use of the drug, and the time at which the drug is administered. No preliminary data are available at this time.

E. Time Course Of The Effects Of THC On Fixed-Interval Responding.

Methodologically, one of the most difficult problems facing researchers in the field of behavioral pharmacology is assessment of the time course of drug action. The technical difficulty of this area of research stems from the need to control a variety of variables which might confound data obtained relative to the time course of drug effects. In the present study, animals were trained to lever press for food delivered on a fixed-interval (FI) schedule of reinforcement. Each subject performed in two separate 30-minute sessions daily. After stabilization of behavior, the animals were administered either THC or vehicle placebo once each week. The test schedule allowed for collection of data from sessions beginning 3, 8, 24 and 80 hours after drug or placebo administration. Response output was generally decreased by the doses of THC employed. The extent of this decrease was dependent on both dose and time elapsed between administration and testing. The lowest three doses had their greatest effect at 24 hours following administration, and the highest dose depressed responding at 30 hours after administration. Because behavior supported by fixed-interval schedules appears to be one of the most sensitive assays for behaviorally active drugs, these data argue strongly for a careful re-assessment of the literature relative to the effects of THC on performance. Failure to recognize the heretofore unexpected duration of behavioral effects of THC may render much of the available data uninterpretable.

F. Time Course Of Oral THC Effects.

As indicated in the study above, in determining the behavioral effect of a drug, time from administration is an important determinant of the results obtained. In the present study, animals were run on a variable-interval (VI) schedule for one-hour sessions. Sessions began 2, 6 or 24 hours following drug administration, and doses of 4, 8, 16 and 32 mg/kg were used. Low doses produced increases in response rate at two and six hours post-administration, but no effect at 24 hours. As a follow-up, animals were run continuously one session every four hours for 65 hours following a dose of 32 mg/kg. Response rate was markedly suppressed at eight hours and recovered sharply between 25 and 30 hours post-drug. These studies are in general agreement with the study reported above in that the behavioral effects of THC appear to be of longer duration than had previously been thought. However, the data also strongly suggest that not all of the effects of the drug endure for the same period of time. With the variable-interval schedule employed in the present study, low dose effects had dissipated within six hours,

while in the fixed-interval study presented above effects were still being obtained at these same dose levels some 24 hours following administration.

G. A Comparison Of Behavioral And Pharmacological Tolerance To THC.

The purpose of this study was to assess the relative contributions of pharmacological tolerance and learning to the concept of behavioral tolerance. Previous work has shown that an oral dose of 32 mg/kg of THC decidedly affects behavior two hours later while not affecting behavior 48 hours later. Well-trained animals running on a Sidman-Avoidance procedure were paired on the basis of their initial response to a 32 mg/kg dose of THC given two hours prior to their work sessions. Subsequently, each member of the pair received THC either two hours or 48 hours prior to the avoidance session. After behavior stabilizes, THC will be administered two hours prior to the final sessions for both groups. Pharmacological tolerance should be comparable because both groups will have received the same number of drug administrations. Thus, any differences between the groups during the final sessions can be attributed to the fact that the two-hour group had an opportunity to practice its performance under the influence of the drug. These results confirm previous observations. During the first THC sessions, all animals avoided fewer shocks than during placebo sessions. This initial disruption in performance has been seen before. However, in the present study it was possible to determine that the increase in shock rate was not correlated with the baseline number of shocks received, indicating that baseline shock rate does not predict initial disruption of avoidance behavior by large doses of THC.

H. Effects Of Chronic THC Administration On Complex Discrimination Behavior.

Two experiments are being conducted in an attempt to develop a behavioral assay that can yield data on the development of pharmacological tolerance to THC without confounding by the concomitant development of behavioral tolerance. In order to develop such an assay, it has been necessary to adopt a strategy of confronting the organism with complex discrimination problems. This is because, in most simple tasks, the organism learns to compensate for any disruptive effect of the drug; thus, the disappearance of drug effects over time are completely confounded with a pharmacological tolerance that might develop and the opportunity of the animal to learn to compensate for the deficit. The

present study takes advantage of the fact that no behavioral compensation occurs for certain kinds of complex discriminations and then exposes the animals to sessions occurring throughout a 24-hour day. A single dose of THC will be administered at the same time each day, and the effect of the drug on each session interposed in the 24-hour period preceding the next drug administration will be examined. Any changes in the time course of the effects of the drug should become apparent with this method. If with repeated administrations of the drug, the duration of a drug effect changes, this change may reflect increase rate of metabolism or some other form of pharmacologic tolerance. To date, no procedure exists that offers the advantages of the present study for the behavioral assay of pharmacological tolerance to drugs. In the first experiment, the animals will work and live unrestrained in cages inside isolation booths. Work panels at the end of each cage contain four levers and four stimulus display units. Sessions will be run around the clock with a session beginning every four hours. A matching-to-sample procedure will be in effect. The stimulus will be complex, that is, a form and a color. On each trial, a sample stimulus will be presented on one of the stimulus display units. Then, all lights will be turned out for 20 seconds. This procedure is referred to as delayed matching-to-sample. Following the delay period, three stimulus choices will be presented to the animal. All three will be colors or all three will be forms. The animal will have to pick the choice that corresponds to the previously displayed sample. This task requires that the animal remember two pieces of information during the 20-second delay interval: the color of the sample and the form of the sample. Correct matches will be followed by food on an intermittent schedule of reinforcement. The second experiment also involves a matching-to-sample procedure, but one of increased complexity. In this procedure, all samples and all choices will be made up of both forms and colors. A tone in the chamber will signal the animal whether to match on the basis of color or on the basis of form. This is known as a conditional discrimination.

V. SELECTIVE BREEDING FOR ALCOHOL INTAKE.

Work continued on the development of rat strains which show differential alcohol intakes under a preferential alcohol drinking test. Production of the fourth filial generation in both the high-drinker and low-drinker strain was completed. The average difference in alcohol intakes between these strains showed a small increase above that recorded for the third generation animals. Fourth generation low-drinkers consumed, on the average, only 5 cc of 10% alcohol per day. There were no intake differences between the males and females. By contrast, high

drinker males consumed 30 cc of the same solution per day and females consumed 25 cc per day. Continued selection should produce even higher alcohol intakes since increases have been achieved with each successive generation. Utilization of these rat strains may facilitate the development of improved laboratory models of chronic alcohol consumption.

Project 3A062110A833 BIOMEDICAL FACTORS IN DRUG ABUSE

Task 00 Biomedical Factors in Drug Abuse

Work Unit 102 Military performance and drug abuse

Literature Cited.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION ^a	2. DATE OF SUMMARY ^a	REPORT CONTROL SYMBOL DD-DR&E(AR)656	
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	A. New	U	U	NA	NL	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	A. WORK UNIT
10. NO. CODES ^a	PROGRAM ELEMENT	PROJECT NUMBER		TASK AREA NUMBER	WORK UNIT NUMBER		
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				NAME ^a Sodetz, CPT R. J.			
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23. (U) This unit examines social, environmental, psychological, and organizational factors that influence the spread of drug abuse, its prevention and treatment.							
24. (U) The methods of clinical psychiatry, social psychology, experimental analysis of behavior, anthropology, epidemiology, physiology, and toxicology are used to identify and modify factors which contribute to drug abuse in the military.							
25. (U) 72 07 - 73 06 Soldiers addicted in RVN possess characteristics which place them in the most prognostically favorable group of addicts. Comparisons of heroin users entering treatment before and after the introduction of urine screening reveal a shorter addiction history and less deviant characteristics in the soldiers referred as a result of chemical detection. Survey of soldier population in Vietnam and in the CONUS reveals that drug users have similar demographic characteristics, but different drug use patterns. They may be characterized as a poly-drug using population who use heroin and other addicting drugs on opportunistically socially determined occasions. The factors influencing the acquisition and behavioral treatment of addiction is under study, using experimental primate systems; analysis of choice behavior in the presence of available heroin is being studied in an environment characterized by qualitatively different reinforcers. The detailed study of the epidemiology of drug abuse at a CONUS army post continues concentrating on explication of the mechanisms of transmission and methods of effectively preventing and treating drug abuse. The analysis of characteristics of a large population of treated Vietnam heroin users continues. For technical report, see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 72-30 Jun 73.							

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Project 3A062110A833 BIOMEDICAL FACTORS IN DRUG ABUSE

Task 00 Biomedical Factors in Drug Abuse

Work Unit 103 Drug abuse prevention in military personnel

Investigators.

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Description.

The purpose of this work unit is to determine those individual, environmental, social and biologic factors which can be modified to decrease the likelihood of the initiation of drug use, disrupt its maintenance, and/or treat its consequences. In order to accomplish this task, information has been collected concerning the social/psychological makeup of drug abusers in Vietnam; a study of the epidemiology of drug abuse in a CONUS military population has been initiated; laboratory studies which examine the environmental contingencies related to maintenance of drug abuse have been evaluated; and the associated neurochemical and physiological changes which are associated with the reinforcing properties of drugs have been studied. In order to obtain a full appreciation of the nature of this effort, it is recommended that the reader consult progress reported under Work Unit 102. (See also 077, 030 and 122).

I. STUDIES IN U.S. ARMY VIETNAM (USARV): THE SOCIO-PSYCHOLOGICAL ASPECTS OF HEROIN USE IN USARV.

Studies related to heroin use by soldiers in USARV were initiated in September 1971. The collection of data and biological specimens pertinent to these studies was terminated in September 1972. In January 1973, a study of the epidemiology of drug abuse among soldiers was initiated at a large CONUS post. Evaluation and analyses of these data and specimens are being carried out at the WRAIR.

A. Demographic characteristics, including personal, family, education, military and illicit drug use histories were collected on 4,000 heroin-using soldiers by means of questionnaire. The same instrument was administered to 3,800 soldiers who denied experience with heroin in the spring of 1972 at DEROS. Seven hundred additional soldiers who admitted to experience with heroin in RVN were also studied at DEROS using the same instrument. This information is currently being prepared for frequency analysis and for cross-tabulated comparisons.

B. Demographic and historical personal data were obtained in a USARV Drug Rehabilitation Center by means of detailed interviews conducted by a physician with 50 heroin-using soldiers.

C. A study to evaluate demographic characteristics of USARV soldiers, both heroin users and non-heroin users, and to further assess their past and present patterns of illicit drug use, attitudes toward illicit drug use and knowledge of illicit drugs, was conducted in July 1972 by use of a self-report instrument designed by COMPSY - WRGH. This study was carried out on 1100 USARV soldiers by LTC Morgan, Dr. Frenkel and MAJ Greden of COMPSY - WRGH and LTC Ream, WRAIR. Initial evaluation of data is completed and is currently being considered for publication.

II. EPIDEMIOLOGY OF DRUG USE.

A. A study of the epidemiology of drug use and abuse at a large CONUS post was initiated during the course of the past year. Phase I of this study involved a mass urine screening procedure, which was completed in March 1973. Over a one-month period, approximately 3208 individual urine specimens were collected from personnel at a CONUS post. In addition to the urine specimens, drug use questionnaires were collected from these persons at the time of the urine screen. The urine specimens have since been analyzed by FRAT and TLC for drug metabolites by the Division of Biochemistry, WRAIR. Data from the urine screen and from the drug use questionnaire have been used to provide an operative model of the patterns of drug use and abuse at the post. Further analysis is presently in progress.

B. The second phase of the project--the collection of mass sociometric information (an aid in determining the possible relationships of friend-

ship patterns to drug use and abuse) was completed during the months of March and April 1973. A sociometric questionnaire was mailed to all enlisted personnel at the post. This questionnaire resulted in a return rate of approximately 35%, better than the expected limits of such rates for mail sociometric questionnaires. The patterns of relationships exhibited by the respondents are presently undergoing analysis.

C. The third phase of the project was also completed in fiscal year 1973. Pursuant to the project protocol, an attempt was made to interview both individuals who had come up as unconfirmed positives for drug use in the urine screen, as well as a sample of urine screen negatives who represented a stratified cross section of the post.

1. All in all, some 460 interviews designed to elicit information on standard demography, social indicators and patterns of drug use, were administered by the team. These data are presently undergoing their initial analysis.

2. A number of preliminary conclusions may be drawn from the initial phases of this research. The data indicate that drug use and abuse, rather than being a diffuse problem equally affecting the post as a whole, are differentially distributed through the various units, companies and detachments stationed at the post. Our convergent information-gathering techniques show that companies are either high, i.e., above 10% in apparent regular drug use, or low, i.e., below 5%. Such use appears to be organized on a company-by-company basis. Drugs are distributed by low-level user-dealers within each military unit and appear to be used with one's fellows from the same company or barracks.

3. The data further indicate that the present "drug scene" involves 20% to 25% of enlisted personnel grade E-5 and under. This population appears to use a number of different drugs on an irregular but recurrent basis. Heroin is the drug of choice for a small majority (no more than 2% of lower ranking enlisted men population). The basic drugs of choice are marijuana and hashish taken with alcohol. In addition to these agents, most other agents, i.e., hallucinogens, soporifics, amphetamines, heroin and cocaine, are used opportunistically when available and when they can be afforded. A further dichotomy may be noted in that drug use is primarily limited to younger troops living in or assigned to barracks. Married enlisted personnel living in quarters or off-post, are a low-risk group insofar as use of drugs other than alcohol is concerned.

a. While areas of the initial data collected have been subjected to preliminary analysis, it is too early to report findings for publication. However, it is clear that polydrug use exists at levels high enough to remain an area of major concern to the Army. The mechanism by which this polydrug use is acquired and spread is being studied by use of direct observation in the field as well as by the use of multiple survey methodologies.

b. Studies of the drug treatment facility and the alcohol treatment facility at a CONUS post are in progress. In addition to the observational procedures, patient and staff personnel are interviewed. The study will provide an in-depth description of the facilities and information relating to the performance of men receiving treatment and the consequences of their treatment.

III. ANALYSIS OF THE CONTINGENCIES UNDERLYING PREVENTION, ESTABLISHMENT, MAINTENANCE AND ELIMINATION OF SELF-ADMINISTRATION OF DRUGS IN LABORATORY PRIMATES.

During the reporting period, a laboratory was established for the behavioral analysis of the problem of drug abuse. Research in that laboratory is directed at identifying those variables which contribute to the establishment and maintenance of drug self-administration, and to using that information to begin a systematic analysis directed at the problem of prevention and elimination of drug self-administration behavior. During the reporting period, 10 baboons were obtained and trained to perform in the laboratory setting and 5 of these have been prepared to self-infuse heroin. Instrumentation permits continuous sampling of blood for radioimmunological assay of blood morphine levels. The five animals that have heroin available in one of three different dose levels are now working to self-administer the drug. In every case, initiation of drug self-administration resulted in a precipitous drop in both food and water intake. No animal has yet shown recovery to pre-drug levels of food and water intake. All three doses of heroin used were effective in establishing drug self-administration behavior. However, self-administration behavior was more readily established in the two animals receiving the highest dose (0.25 mg/kg per infusion). In one animal, infusion rates totaling 500 mg in a 24-hour period were observed within five days of initial exposure to the drug. A second animal is reliably administering between 250 and 400 mg/kg/day, fewer than 20 days following initial exposure to the drug.

IV. EFFECTS OF PERFORMANCE DEMANDS ON HEROIN SELF-ADMINISTRATION.

Two monkeys are performing in a procedure for which lever presses produce intravenous infusions of .05 ml/kg of heroin. To date, one animal has been run through an ascending sequence of ratios from 1 to 240. The highest ratio did not entirely eliminate heroin intake, although it was reduced to about 20% of the fixed ratio one level. The two current objectives of this study are: (1) to ascertain the degree to which heroin self-administration can be modulated by manipulating the number of responses required to produce an infusion; and (2) to compare heroin-reinforced and morphine-reinforced behavior in an effort to establish a full link to the body of literature available on morphine reinforcement.

V. ESTABLISHMENT OF ORAL HEROIN SELF-ADMINISTRATION.

The bulk of the data and literature related to morphine has been developed in studies of the laboratory rat. To date, no method has existed for producing reliable self-administration of either morphine or heroin in these animals. During the reporting period, a method was developed for inducing ingestion of large amounts of heroin by rats. The need for tedious injection regimens, elaborate instrumentation for venous cannulation, or surgical trauma for the rat have all been alleviated by this method. Large numbers of animals can be cared for easily and the rats regulate their own daily intake of heroin. The final version of the method involves a recycling three-day sequence, two days of which the only fluid available to the animal is a 0.10% solution of sodium saccharine, containing 0.17 gm heroin per milliliter followed by a choice day, during which two bottles are simultaneously available, one containing saccharine solution and one containing saccharine solution with heroin added. Initial reaction to saccharine solution is usually a two- to three-fold increase in daily fluid intake. Intake typically falls to near zero when heroin is added, and rats have overwhelmingly preferred the plain saccharine solution to the drug solution. Within a week, there is some recovery of fluid intake on single bottle days; however, several weeks are required before substantial amounts of heroin are taken on choice days. Neither of these procedures were speeded by prior or concurrent intraperitoneal injections of heroin. Eight percent of rats tested thus far have ultimately come to take a majority of their choice-day fluid in the form of heroin solution. The portion of total fluid intake taken as a heroin solution also served as a highly reliable prediction of weight loss on subsequent withdrawal from the drug. The amount of heroin solution consumed showed only a modest correlation

with weight loss. In another experiment, a 30-day period of enforced abstinence eliminated drug preferences in addicted animals. Preferences were successfully reestablished, but no faster than they were established the first time or in controls treated identically to this point except for the use of plain saccharine solution on single bottle day.

VI. HEROIN AND CONDITIONED AVERSION.

An argument frequently proposed to account for the difficulty in managing the abuse of heroin has involved the claim that heroin has inherent reinforcing properties. The conditioned aversion technique offers a means of evaluating the aversiveness of an exposure to an agent, such as any abuse compound. Recent studies in this laboratory have demonstrated that THC, methamphetamine, pentabarbital and ethanol, all are capable of inducing a conditioned aversion which suggests that these compounds do not have inherently reinforcing properties. However, heroin, even at doses 32 mg/kg, administered intraperitoneally to laboratory rats, failed to produce a significant aversion even though studies in other laboratories have demonstrated an aversion to morphine. A second study is in progress, attempting to evaluate whether route of administration would be the critical variable in determining the failure to obtain a conditioned aversion. It is important to evaluate this methodological consideration before drawing any conclusions regarding differences in the reinforcing properties of abuse compounds.

VII. DRUGS OF ABUSE ON BRAIN CHEMISTRY AND ON BEHAVIOR.

A. Studies are in progress on the mechanism of action of various drugs of abuse on brain chemistry and on behavior. These include the acute and chronic effects of opiates, stimulants, alcohol, and barbiturates on neurotransmitter chemicals in brain and cerebrospinal fluid. Secondly, the pharmacological modification of pathological behavior is being evaluated. Research methods employed include neurochemistry, neuroanatomy, pharmacology, neuroendocrinology and physiological psychiatry. Specific approaches include:

1. Effects of drugs of abuse on intracranial self-stimulation.
2. Effects of drugs of abuse on neurotransmitter changes in brain regions.
3. Cerebrospinal fluid neurotransmitters and metabolites as monitors of CNS effects of acute and chronic administration of drugs of abuse.

4. Protein synthesis pattern in brain as indicator of development of tolerance.

5. The differential behavioral effects of optical isomers of drugs of abuse.

B. An experiment has been completed demonstrating that daily injections of heroin produce a 300% increase in intracranial self-stimulation (ICSS) by the fifth injection. The latency of onset of effect decreases with successive injections. Pre-treatment with naloxone, a specific opiate antagonist attenuates the heroin-induced facilitation by 75%. Daily saline injections produced no change in ICSS. In this experiment food and water intake remained unchanged during the course of heroin administration. This work has been done in collaboration with CPT George Koob and Dr. N. Herbert Spector, Chief, Department of Neurophysiology.

C. A number of projects have been initiated to test the effects of drugs of abuse on neurotransmitters and cyclic nucleotides in specific brain regions. A method has been established which permits assay of gamma-aminobutyric acid (GABA), glutamic acid (GLU), cyclic adenosine 3'5' monophosphate (cAMP), and cyclic guanosine 3'5' monophosphate (cGMP) in the same sample of brain tissue after microwave inactivation of enzymes, thereby increasing the amount of information obtainable from a single experiment. Two experiments demonstrated that cGMP is markedly decreased in brainstem following acute heroin administration. GABA and GLU remain unchanged. This is significant in that cGMP is thought to be involved in cholinergic transmission (Ferrendelli, 1972) and there are indications that opiates impede release of acetylcholine (Beleslin, 1965).

1. GABA and GLU are considered to be, respectively, inhibitory and excitatory transmitters (Krnjevic, 1966). The tentative implication (Schumann, 1962) of GABA deficiency in convulsions suggests that it will be an important variable to monitor in studies involving administration of ethanol or barbiturates. Under various conditions, brain tissue cAMP is stimulated by norepinephrine, dopamine, serotonin and histamine (Huang, 1972; Brown, 1972). Within the next two years, emphasis in the field will shift to cAMP/cGMP ratios, and the capacity to study both will become essential.

2. A new assay for catecholamines (CA) has been set up which has one hundred times the sensitivity of other methods (Coyle, 1973). This enzymatic-radioisotope method permits reliable assay of CA assay as little as 1×10^{-9} gram of brain tissue, and makes it possible to study changes in CA levels and turnover in discrete brain regions. Previous studies of effect of drugs of abuse on transmitter turnover have not been able to focus on discrete brain regions. In addition, the extreme sensitivity of this assay may permit measurement of NE and DA in csf where existing techniques have failed.

D. Cerebrospinal fluid neurotransmitters and their metabolites as monitors of effects on brain of acute and chronic administration of drugs of abuse.

1. Logistic and technical problems have been solved and we are now sampling cerebrospinal fluid (csf) continuously, collecting hourly samples over successive 24-hour periods. Studies are in progress to determine baseline parameters and possible circadian or ultradian variations prior to initiating drug studies. We are monitoring csf levels of several neurotransmitter metabolites:

a. Homovanillic acid (HVA)-major metabolite of dopamine (Ashcroft, 1968).

b. Five-hydroxyindole acetic acid (5 HIAA)-major metabolite of serotonin, (Ashcroft).

c. Three-methoxy, four-hydroxyphenylethylglycol (MHPG)-major metabolite of norepinephrine in brain (Schanberg, 1968; Gordon, 1971).

2. The csf monitoring model has the advantage that each subject can be followed through a course of acute and chronic drug administration and acute abstinence. cAMP and cGMP levels in csf will also be monitored.

3. Recently completed studies include determination of storage parameters of 5 HIAA and HVA under various experimental collecting conditions, and establishing a csf pool to control for interassay reliability. We have determined that at 25°C, csf values do not change over a 24-hour period and at 4°C they are unchanged after one week. Ascorbic acid is not required for storage up to four months at -70°C. Standards in water can be stored no longer than one week. Recent experiments have demonstrated that ascorbic acid interferes with the assay. This is significant in

that some published methods routinely collect csf with ascorbic acid added before storage (Gordon, 1971). Csf levels of cAMP and cGMP have been shown to be stable up to 24 hours at 25°C.

E. Protein synthesis pattern in brain as indicator of development of tolerance to drugs of abuse.

Recently completed work studies the pattern of incorporation of amino acids into polypeptides by brain polysomes. Since development of tolerance to opiates is blocked by cyclohexamide (Loh, 1970), an inhibitor of protein synthesis, it is hypothesized that the phenomenon of tolerance may be mediated by a process requiring protein synthesis. Current experiments are examining the effect of opiates on incorporation ratios of leucine and phenylalanine into polypeptide.

F. Differential effects on behavior of optical isomers of amphetamine.

In collaboration with Dr. Solomon Snyder of Johns Hopkins University, a study was completed on effects of amphetamine isomers on Gilles de la Tourette's disease, a tic disorder that often involves compulsive uttering of obscenities and on the hyperactivity associated with minimal brain dysfunction. d-Amphetamine markedly increased the frequency of tics while l-Amphetamine did not alter the tics. By contrast, both isomers decreased the patient's hyperactivity to a similar extent.

Project 3A062110A833 BIOMEDICAL FACTORS IN DRUG ABUSE

Task 00 Biomedical Factors in Drug Abuse

Work Unit 103 Drug abuse prevention in military personnel

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION ^a	2. DATE OF SUMMARY ^a	REPORT CONTROL SYMBOL ^a	
				DA OB 6494	73 07 01	DD-DR&E(AR)036	
3. DATE PREV SUMMARY	4. KIND OF SUMMARY	5. SUMMARY SCTY ^a	6. WORK SECURITY ^a	7. REGRADING ^a	8a. DISSEM INSTR ^a	8b. SPECIFIC DATA - CONTRACTOR ACCESS ^a	9. LEVEL OF SUM ^a
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10. NO./CODES ^a		PROGRAM ELEMENT		PROJECT NUMBER		TASK AREA NUMBER	
a. PRIMARY		63713A		3A0672110A833		00 104	
b. CONTRIBUTING							
c. CONTRIBUTING		CDOG 114(f)					
11. TITLE (Precede with Security Classification Code) ^a							
(U) Drug Tests Systems							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS ^a							
002600 Biology							
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
72 07		CONT		DA		In-House	
17. CONTRACT/GRANT				18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	
a. DATES/EFFECTIVE: NA				PRECEDING		b. FUNDS (in thousands)	
b. NUMBER ^a				FISCAL		73 3 105	
c. TYPE				YEAR		CURRENT	
d. KIND OF AWARD:				74		3 105	
19. RESPONSIBLE DOD ORGANIZATION				20. PERFORMING ORGANIZATION			
NAME ^a Walter Reed Army Institute of Research				NAME ^a Walter Reed Army Institute of Research			
ADDRESS ^a Washington, D.C. 20012				Division of Medicinal Chemistry			
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TELEPHONE: 202/576-3551				TELEPHONE: 202/576-2292			
21. GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER: [REDACTED]			
Foreign Intelligence Not Considered				ASSOCIATE INVESTIGATORS			
				NAME: Atherton, B. T., 1LT			
				NAME: Wolf, C. R., 2LT DA			
22. KEYWORDS (Precede EACH with Security Classification Code) (U) Antagonists; (U) Drug Abuse; (U) Drug Addiction; (U) Barbiturates; (U) Amphetamines; (U) Drug Dependence							
23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
<p>23. (U) To develop test systems and test compounds which act as antagonists to drugs for which a tolerance has been developed.</p> <p>24. (U) The use of methadone and cyclazocine to maintain or antagonize the pharmacodynamic effects of opioid addiction point to the potential application of pharmacological antagonists of drugs of abuse in the treatment of addiction.</p> <p>25. (U) 72 07 - 73 06 The problem of drug addiction is a great problem in the U.S. Army. Although no clinical applicable antagonists have been developed for use in treating barbiturate or amphetamine dependence, demonstrations of antagonisms have been successful. An extensive literature search has been conducted. The methods of approach to the problem will involve the use of selective screening of existing compounds in animal test models. These models will be a variation of the systems used to demonstrate tolerance and dependence potentials of new drugs. Development and standardization of these test systems are considered as an integral part of the total scope of this project. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 72 - 30 Jun 73.</p>							

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DD FORM 1498

PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A 1 NOV 65 AND 1498-1 1 MAR 66 (FOR ARMY USE) ARE OBSOLETE

Project 3A062110A833 Biomedical Factors in Drug Abuse

Task 00 Biomedical Factors in Drug Abuse

Work Unit 104 Drug Test Systems

Investigators:

Principal: LTC Kenneth E. Kinnamon, VC

Associate: 1LT Blair T. Atherton, MSC

The use of methadone and cyclazocine to maintain or antagonize the effects of opioid addiction point to the potential application of pharmacological antagonists of drugs of abuse in the treatment of drug addiction. Though no clinically applicable antagonists have been developed for use in treating barbiturates or amphetamine dependence, laboratory demonstrations of such antagonisms have been successful. The foregoing provides the practical justification for attempting to develop new drugs for potential clinical use to antagonize dependence on drugs of abuse.

An extensive literature search has been conducted. The methods of approach to the problem will involve the use of selective screening of existing compounds in animal test models. These models will be a variation of the systems used to demonstrate tolerance and dependence potentials of new drugs. Development and standardization of these test systems are contemplated as an integral part of the total scope of this project.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1 AGENCY ACCESSION	2 DATE OF SUMMARY	REPORT CONTROL SYMBOL	
				DA OB 6498	73 07 01	DD-DR&E(AR)836	
3 DATE PREVIOUS SUMMARY	4 KIND OF SUMMARY	5 SUMMARY SCTY	6 WORK SECURITY	7 REGRADING	8A DISEN INSTRN	8B SPECIFIC DATA CONTRACTOR ACCESS	9 LEVEL OF SUMMARY
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B. CONTRIBUTING							
C. XXXXXXXX	CDOG 114(f)						
11 TITLE (Precede with Security Classification Code)							
(U) Cellular Aspects of the Metabolism of Drugs of Abuse							
12 SCIENTIFIC AND TECHNOLOGICAL AREAS							
012600 Pharmacology; 012900 Physiology; 016800 Toxicology							
13 START DATE		14 ESTIMATED COMPLETION DATE		15 FUNDING AGENCY		16 PERFORMANCE METHOD	
72 10		CONT		DA		C. In-House	
17 CONTRACT GRANT				18 RESOURCES ESTIMATE		19 PROFESSIONAL MAN YRS	
NA				PRECEDENCE			
A. DATES/EFFECTIVE				FISCAL YEAR		2	
B. NUMBER				73		125	
C. TYPE				74		2.9	
D. KIND OF AWARD						94	
E. AMOUNT							
F. CUM. AMT.							
19 RESPONSIBLE DOD ORGANIZATION				20 PERFORMING ORGANIZATION			
NAME: Walter Reed Army Institute of Research				NAME: Walter Reed Army Institute of Research			
ADDRESS: Washington, D.C. 20012				ADDRESS: Washington, D.C. 20012			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)			
NAME: Buescher, COL E.L.				NAME: Glinos, A.D., MD			
TELEPHONE: 202-576-3551				TELEPHONE: 202-576-5284			
				SOCIAL SECURITY ACCOUNT NUMBER			
21 GENERAL USE				ASSOCIATE INVESTIGATORS			
Foreign Intelligence Not Considered				NAME: Bartos, E.M., Ph.D.			
				NAME: Vail, J.M., Ph.D.			
				DA			
22 KEYWORDS (Precede EACH with Security Classification Code)							
(U) Acetylcholinesterase Regulation; (U) Cell Culture; (U) Cellular Pharmacology; (U) Morphine Tolerance; (U) Phenotypic Reprogramming.							
23 TECHNICAL OBJECTIVE, 24 APPROACH, 25 PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code)							
<p>23. (U) To investigate the alterations of cellular metabolism underlying the development of tolerance to and dependence on drugs of abuse.</p> <p>24. (U) A cell culture system with well defined density dependent physiological responses is used in reproducing and analyzing selected actions of narcotic drugs.</p> <p>25. (U) 72 10 - 73 06 Continuous exposure of WRL-10A mouse fibroblasts to morphine sulfate elicited a dose dependent response involving generation time (T), cytopathology and viability. Thus, with .25mM morphine the only effect was a transient increase of T from 24 to 28 hrs; with .50mM, T rose to 46 hrs while the cells exhibited progressive granularity, vacuolation, irregular surface and variable size and shape with cell death occurring at 47 days; with 1.0mM, T was extended almost indefinitely, there was pronounced early cytopathology and cell death occurred at 14 days. When cells cultured in the presence of .25mM morphine for 4 months were shifted up to .50mM only, the transient increase of T to 28 hrs was noted and a further shift up to 1.0mM morphine 6 months later showed the cells viable at 40 days with T=41 hrs and only slight granularity and vacuolation. These results indicate development of tolerance to morphine and suggest the possibility that WRL-10A fibroblasts contain receptors normally present only in neuronal and related cells. It was found that acetylcholinesterase (AChE) is present in small amounts in low density exponentially growing cultures of WRL-10A cells and increases 100X in high density populations where DNA synthesis and cell division are inhibited. Regulation of AChE in this fashion has hitherto been described only in nerve and muscle cells; its presence in cells of fibroblastic origin indicates phenotypic reprogramming. This finding is currently exploited in investigating the possible role of the cholinergic system in the development of tolerance to morphine. For technical reports see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 72 - 30 Jun 73.</p>							

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PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DO FORMS 1498A, 1 NOV 68 AND 1498 I, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE.

1274

Project 3A062110A833 BIOMEDICAL FACTORS IN DRUG ABUSE

Task 00 Biomedical Factors in Drug Abuse

Work Unit 105 Cellular aspects of the metabolism of drugs of abuse

Investigators.

Principal: Andre D. Glinos, M.D.

Associate: Edwin M. Bartos, Ph.D.; Robert J. Werrlein, M.S.;
Richard C. Robinson, B.A.; James M. Vail, Ph.D.

Description

Attempts to reproduce the phenomena of drug tolerance and dependence in cultured cells thus paving the way for the eventual uncovering of the metabolic processes involved in drug abuse are not new. Beginning with the prewar heroic period of tissue culture¹ and up to the present time² a sizeable number of reports on the subject has accumulated with an approximately equal distribution of positive and negative results. Thus, it is characteristic that in the course of the last year one group of investigators reported that levorphanol prevented the induction of acetylcholinesterase (AChE) in cultures of mouse neuroblastoma cells without development of either tolerance or dependence², a second group claimed that morphine sulfate induced a multifold increase of AChE with development of limited tolerance and total dependence in cultures of human neuroblastoma³, while a third found that morphine sulfate increased AChE activity by only 50% and induced tolerance but no dependence in cultured chick embryo brain cells⁴. The multitude of cell types, culture methods, treatment schedules and observational criteria used as well as the rather large variance inherent in long-term linear tissue culture experiments are undoubtedly responsible for these inconsistencies. It follows that to answer unequivocally the question as to whether it is possible to reproduce the phenomena of drug tolerance and dependence *in vitro* there is an urgent need to use a well characterized cell culture system in conjunction with a rigorously standardized methodology. At this point, the system does not need to be neuronal as opiate tolerance and dependence have been reported in other cell types⁵ with no greater inconsistency⁶ than described above for neuronal cells. Accordingly, we undertook a feasibility study of tolerance to morphine in cell culture using L-929 mouse fibroblasts.

Progress and Results

The origin and karyology of L-929, clone WRL-10A cells and their utilization in developing a suspension culture system with well

defined density dependent metabolic regulation have been described previously^{7,8,9,10}. To determine the sensitivity of the system to morphine, 50 ml suspension cultures of WRL-10A cells were maintained at population densities below 10^6 cells/ml through daily centrifugation and resuspension of the appropriate number of cells in fresh media. The growth of these cultures was estimated on the basis of cell counts performed at the beginning and the end of the daily media renewal cycle and expressed either as the factorial increase or the generation time of the cell population.

In a first series of experiments, morphine sulfate was added to the media of the cultures during their daily renewal at a final concentration ranging from 10^{-4} to 10^{-3} M. It was found that the drug affected the growth rate, morphology and viability of the cells, in that order, to a degree which depended on its concentration. Thus, at 10^{-4} M there was no measurable effect of the drug on any of the three parameters mentioned, at $.25 \times 10^{-3}$ M there was only a 20-30% depression of the daily factorial increase of the population, while at $.50 \times 10^{-3}$ M the daily factorial increase was depressed by approximately 50% and the cells showed slight cytoplasmic granularity, formation of small vacuoles, cell membranes with somewhat frayed appearance and variable cell size and shape. These morphological changes are typical manifestations of cytotoxicity and were classified as cytopathology Grade I. At 10^{-3} M the daily factorial increase of the population was depressed by approximately 90% and the morphological changes being considerably more pronounced were classified as cytopathology Grade III.

During the first 3 days of exposure to morphine, the viability of the cells, as evaluated by the uptake of nigrosin, remained normal and all the effects described above were readily reversible upon withdrawal of the drug. Prolongation of exposure beyond this time, however, resulted in marked differences among the cultures. Thus, while there was no effect on cell morphology and viability at the 0.25mM level, cultures exposed to .50 and 1.0mM morphine showed progressively increasing cytopathology eventually reaching Grade IV, characterized by extreme granularity and vacuolation of the cytoplasm, ectopic nuclei, ruptured cell membranes, cell clumping and ghost cell formation. This progression was accompanied by decreased viability indicated by increase of the fraction of cells staining with nigrosin and decrease of the cell counts ending with the death of the entire population. The rate of this progression was proportional to the concentration of the drug in the media, the mean survival time being 47 and 14 days for the .50 and 1.0mM morphine levels, respectively.

It may be seen that if the daily factorial increase of the cell population is plotted as a function of the concentration of morphine as in Fig. 1, a typical log dose-response curve is obtained.

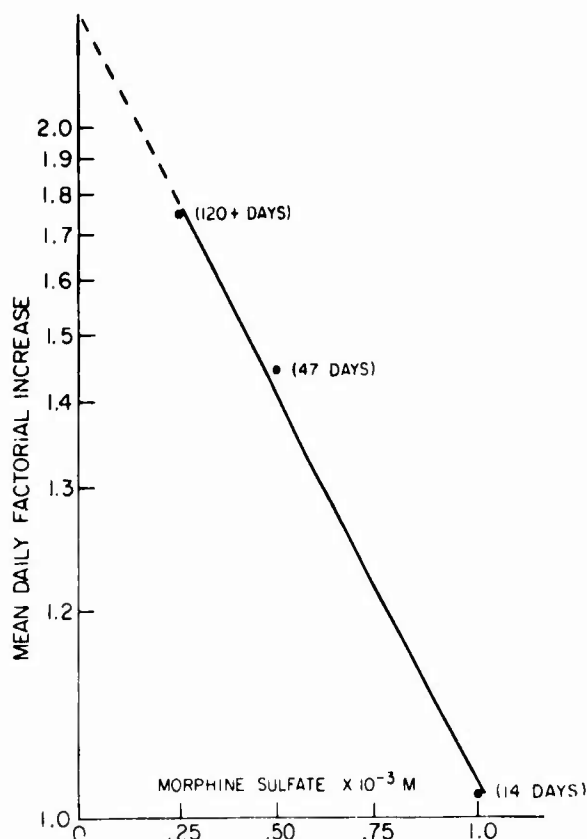


Figure 1 - Effect of morphine on the growth of low density suspension cultures of WRL-10A cells. The factorial increase of the cell populations was determined daily as described in the text and averaged over the time intervals indicated in parentheses. In the case of the .50 and 1.0mM concentrations these intervals represent also total survival time of the cultures.

When the curve is extrapolated below the .25mM concentration, it intersects the factorial increase ordinate at a point between 2.0 and 2.5 which corresponds to a generation time of 18 - 24 hours and is characteristic of low density WRL-10A cultures growing exponentially in the absence of morphine. This typical quantitative relationship between drug concentration and growth indicates that the cell culture system used is well suited for studying effects of opiates at the cellular level and that changes in the sensitivity of the cells to these drugs may be readily detected by using the daily factorial increase of the population as an indicator.

Based on these findings a long-term linear study of low density suspension cultures of WRL-10A cells exposed to progressively increasing concentrations of morphine sulfate was undertaken. To protect

against accidental losses cell cultures were set up in duplicate, incubated separately and treated by different individuals. To initiate the study, cells exposed to .25mM morphine for 104 days were subcultured at a concentration of .50mM morphine and factorial increase, cell morphology and cell viability determined daily and compared with similar data obtained from cultures continuing at the .25mM level. After exposure to the .50mM level for 183 days the cells were subcultured again in media containing 1.0mM morphine. To determine the growth response of the cells after a drug concentration shift up, the daily factorial increase of the population was averaged for a period of one month beginning immediately after the shift up and again at a desired later time interval. The cytopathology considered to be associated with the factorial increase means thus obtained was the highest grade observed within the corresponding time intervals. The results obtained as of the writing of this report are shown in Table 1.

Table 1 - Development of tolerance to the toxic effects of morphine in low density suspension cultures of WRL-10A cells.

Dose (mM Morphine Sulfate)	Exposure (days)	Daily Fact. Increase	Cytopathology Grade
.25	120	1.76	-
	330	2.03	-
.50	60 (+104 at .25mM)	1.74	+
	230 (+104 at .25mM)	1.98	-
1.00	40 (+183 at .50mM +104 at .25mM)	1.5	++
	No data available beyond 40 days.		

Figures in parentheses indicate times of pre-exposure at the indicated molar concentrations of morphine. Factorial increase figures were obtained by averaging the daily determinations over a period of at least one month. Cytopathology graded as described in the text.

Consideration of the data in the table in conjunction with the findings obtained in the first series of experiments and summarized in Fig. 1

indicates that: a) after prolonged exposure of the cells to a given drug concentration the growth rate of the cells returns to control levels; b) the depression of the daily factorial increase and cytopathology characteristic for a given drug level are considerably attenuated if the cells have been pre-exposed to a lower concentration of the drug; and c) at lethal concentrations of morphine, survival time is markedly increased if the cells have been pre-exposed to a non-lethal drug level. These findings indicate that cultures of WRL-10A cells exposed continuously to morphine sulfate develop tolerance to the toxic effects of the drug. As these cultures involved low density exponentially growing populations there are no indications as to whether the development of tolerance was due to functional adaptation or to selective propagation of a small drug resistant fraction of the original cell population. This question will be answered through future experimentation involving high density growth inhibited populations with well defined metabolic characteristics^{8,9,10}.

Regardless of the evolutionary process involved, the fact that tolerance to morphine did develop (Table 1) and that the dose-response relationship follows first order kinetics (Fig. 1) is most suggestive from the viewpoint of the actual metabolic interactions underlying the effects of the drug. Because of these findings, it appeared of the utmost importance to investigate whether WRL-10A cells, although of fibroblastic origin, contain metabolic components which normally would be expected to be related to the morphine receptor in neuronal cells. Acetylcholinesterase (AChE) appeared to be a good candidate in this respect for the following reasons: 1) recent findings suggest that the morphine receptor *in vivo* is very likely associated with the cholinergic system¹¹; 2) as previously mentioned, the evidence regarding the behavior of AChE in cultures of neuronal cells treated with morphine is entirely inconsistent^{2,3,4}; and 3) AChE activity in neuronal cells is growth regulated, increasing approximately 30X in dense cultures of neuroblastoma where DNA replication, mitosis and total protein synthesis are markedly inhibited¹². This last feature provides an excellent test for any possible similarity in regard to AChE between WRL-10A cells and neuroblastoma because DNA replication, mitosis and total protein synthesis in the former are density inhibited precisely as in the latter. Accordingly, high density, growth inhibited populations of WRL-10A cells were produced by omitting cell dilution at the time of daily medium renewal. Cultures treated in this manner increased to characteristic population densities of $6 - 10 \times 10^6$ cells/ml within 10 days, and thereafter maintained a relatively stable high cell population. At desired times and depending on cell density, samples with the desired number of cells were obtained from the cultures and centrifuged at $300 \times g$ for 15 min. The cell pellets were subsequently washed three times in Earle's balanced saline and resuspended in 1.5 ml of phosphate buffer (50 mM potassium phosphate buffer, pH 6.8; 1mM EDTA, potassium salt). The samples were then either stored at -80° or sonicated (Sonifier Cell Disruptor, Heat Systems Ultrasonics, Inc.) for

10 sec and assayed. All procedures during preparation of the samples were carried out at 5°; there were no differences in the activities of samples sonicated and assayed immediately and of those stored prior to assay.

Samples were assayed for AChE as described by Wilson et al.¹³ using ¹⁴C acetylcholine iodide (4.54 mCi per mmole; New England Nuclear) as substrate and incubating the reactions for 30 minutes at 37°. Each sample was assayed at 4 concentrations and the values obtained were considered valid only if proportional to sample concentration. The specificity of the reaction was evaluated by the activity remaining after adding BW284C51 dibromide, a powerful inhibitor of AChE.

The protein content of the samples was determined through conventional methods. One unit of AChE activity was defined as 1.0 nmol ¹⁴C acetate per mg protein.

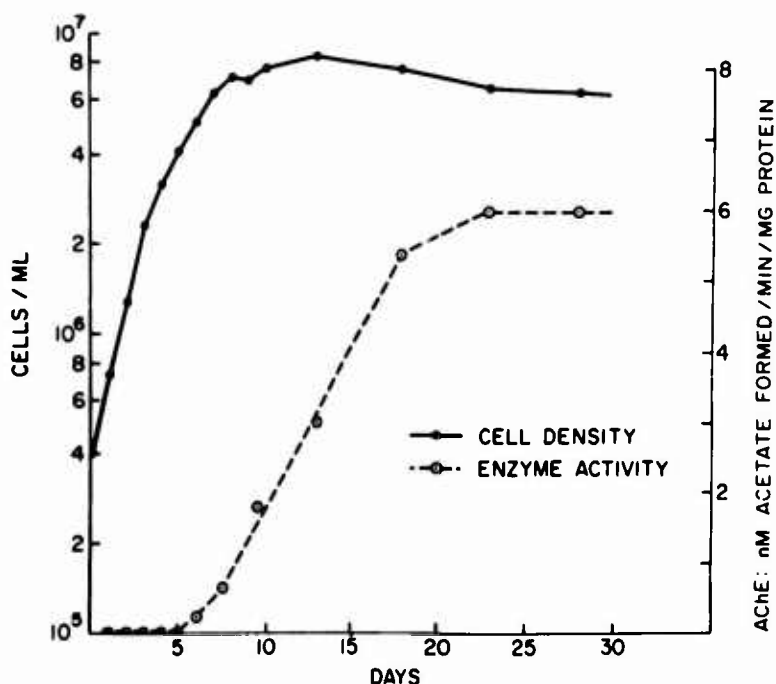


Figure 2 - Cell population kinetics and AChE specific activity during the establishment of high density WRL-10A cell suspension cultures. The data shown represent mean values obtained from 5 different cultures grown for 30 days. Cell counts have been plotted daily during the exponential and near exponential growth phase and as 5-day averages for the high density population. AChE specific activity has been determined daily for the first 5 days. After this time the cultures were sampled at variable and mutually complementary times, the AChE specific activity determined and 2, 3 and 5-day averages calculated and plotted as shown.

The specific activity of AChE in exponentially growing cultures kept at low density through daily dilution and sampled for 3 consecutive days ranged from 0.016 to 0.032 with a mean value of .025 units. When the cells were not diluted and the density of the cultures started to increase as shown in Fig. 2, the specific activity of the enzyme remained within this range for the first 3 days of exponential growth. During the fourth and fifth days, however, when the growth rate began to decline the mean specific activity of the enzyme increased to .063 with a range of .035 to .118 units. As these values are all in the vicinity of the lower limit of the sensitivity of the assay the activity of exponentially and near exponentially growing cultures has been indicated on the graph simply as a stable baseline level of approximately .05 units.

There was a significant rise of enzyme activity on day 6 and beginning with day 7 a steep linear increase coincidental with the termination of the growth phase of the cultures and the establishment of the high density populations. This increase continued until day 23 when the specific activity of AChE leveled off at a mean value of 6.03 and a range of 2.38 - 13.18 units. As reported earlier⁸ the transition from exponential growth to the high density phase did not alter the amount of total protein per cell which in the current experiments averaged 280 pg.

The possibility was considered that the source of AChE activity exhibited by WRL-10A cells in these experiments was the horse serum in the media. It was found that while the enzyme activities of freshly made unused media and of media obtained from low density cultures was nearly identical, the activity of media obtained from high density populations was significantly higher. This indicates release of enzyme from the cells into the media comparable to the release known to occur from muscle cells.

The possibility that the observed differences between low and high density populations were due to changes in nonspecific esterases was also investigated. After use of BW284C51 as described previously the residual activity of homogenates from exponentially growing cultures (Fig. 2, 1-3 days) was .04 nmol ¹⁴C acetate formed/min/mg protein, or 64 percent of the total activity prior to the addition of the inhibitor, while the residual activity of homogenates from high density cultures (Fig. 2, 21-30 days) was .12 nmol ¹⁴C acetate formed/min/mg protein, or 2.4 percent of the total. On this basis, and pending further identification of the enzyme, it appears most likely that differences in the ability of homogenates from high and low density cultures of WRL-10A cells to catalyze acetylcholine hydrolysis are due to differences in the activity of AChE (EC 3.1.1.7).

The possibility that the differences of the AChE specific activity were due to dissociable activators or inhibitors of the enzyme was

excluded since all mixing and dilution experiments using homogenates of exponentially growing and high density cultures gave the predicted additive results.

Finally, the possibility was considered that the rise of the AChE specific activity was merely an expression of the lack of net protein synthesis in the high density cultures. During exponential and near exponential growth the doubling time of the cells was 27 hours (Fig. 2), the G₁ phase approximately half this time, and the specific activity of the enzyme .05 units. Assuming that AChE is normally synthesized in the G₁ phase and that in high density cultures the entire population is arrested in that phase, it may be calculated that with no change in either the rate of synthesis or the rate constant of degradation of the enzyme, its specific activity should increase with a maximal rate of .08 units per day. The actual rate indicated by the slope of the linear portion of the curve in Fig. 2 is .44 units per day. This indicates that the increase of the activity of AChE cannot be ascribed simply to extended G₁ phase synthesis and accumulation of the enzyme in the absence of net protein increase. A more likely possibility is that the marked depression of protein synthesis in the high density cultures¹⁰ affects proteins which in actively growing, low density cultures block the expression of AChE activity at the transcriptional or post transcriptional level; the alternative possibility that these proteins participate in the degradative phase of AChE turnover must also be considered.

It is known that AChE activity is inversely related to growth in nervous and muscle tissue during normal development and in neuroblastoma cells during the transition from the dividing to the nondividing state¹². In contrast, fibroblasts, whether normal diploid, transformed but contact inhibited, or, derivatives of the L-929 line, have been found so far to exhibit only minimal amounts of AChE even when grown to high cell densities in attached cultures¹³. Consequently, this is the first time that cells of connective tissue origin have been shown to exhibit regulation of AChE. In fact, the increase of AChE specific activity in high density WRL-10A cell suspension cultures reported here is even greater than that reported for neuroblastoma cells¹².

Although originating in the subcutaneous connective tissue of a newborn mouse, L-929 cells and its derivative WRL-10A clone used in these experiments, represent a long-established, transformed cell line. It is, therefore, most likely that the regulation of AChE activity demonstrated in this report is an expression of partial reprogramming of fibroblasts toward the phenotype of muscle cells. This finding is currently exploited in investigating the possible role of the cholinergic system in the development of tolerance to morphine by WRL-10A cells described in the first part of this report.

Summary and Conclusions

Continuous exposure of WRL-10A mouse fibroblasts to morphine sulfate elicited a dose dependent response involving generation time (T), cytopathology and viability. Thus, with .25mM morphine the only effect was a transient increase of T from 24 to 28 hrs; with .50mM, T rose to 46 hrs while the cells exhibited progressive granularity, vacuolation, irregular surface and variable size and shape with cell death occurring at 47 days; with 1.0mM, T was extended almost indefinitely, there was pronounced early cytopathology and cell death occurred at 14 days. When cells cultured in the presence of .25mM morphine for 4 months, were shifted up to .50mM only the transient increase of T to 28 hrs was noted and a further shift up to 1.0mM morphine 6 months later shows the cells viable at 40 days with T=41 hrs and only slight granularity and vacuolation. These results indicate development of tolerance to morphine and suggest the possibility that WRL-10A fibroblasts contain receptors normally present only in neuronal and related cells. It was found that acetylcholinesterase (AChE) is present in small amounts in low density exponentially growing cultures of WRL-10A cells and increases 100X in high density populations where DNA synthesis and cell division are inhibited. Regulation of AChE in this fashion has hitherto been described only in nerve and muscle cells; its presence in cells of fibroblastic origin indicates phenotypic reprogramming. This finding is currently exploited in investigating the possible role of the cholinergic system in the development of tolerance to morphine.

Project 3A062110A833 BIOMEDICAL FACTORS IN DRUG ABUSE

Task 00 Biomedical Factors in Drug Abuse

Work Unit 105 Cellular aspects of the metabolism of drugs of abuse

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